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Role of bHLH93 in Controlling Flowering Time in *Arabidopsis thaliana*

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Role of bHLH93 in Controlling Flowering Time in *Arabidopsis thaliana*

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My loving mother

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Role of bHLH93 in Controlling Flowering Time in *Arabidopsis thaliana*

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In plants, flowering time is a tightly regulated process where several environmental and endogenous cues fine-tune the time of flowering. In *Arabidopsis*, four major genetic pathways regulate flowering time, namely photoperiod, vernalization, autonomous, and phytohormone gibberellic acid (GA) pathways. *Arabidopsis* is a facultative long day (LD) plant. LD promotes flowering whereas flowering is delayed in short day (SD) conditions. Here, we identified a **b**asic-**h**elix-**l**oop-**h**elix (bHLH) transcription factor called bHLH93 that is necessary to promote flowering only in SD. Also, photoperiod plays more critical roles in regulation of flowering time of *bhlh93* mutant compared to GA and vernalization pathways. Thus, bHLH93 might represent a novel transcription factor absolutely required for *Arabidopsis thaliana* to evolve as a facultative LD plant. *bhlh93* mutants also show severe adult phenotype such as shorter stature, curly and darker green leaves, and reduced fertility compared to wild type plants. These results suggest that bHLH93 controls plant stature, fertility and chlorophyll content in *Arabidopsis*. *bHLH93* is expressed in a tissue-specific and developmental stage-dependent manner. bHLH93-YFP protein is localized in the nucleus. bHLH93 homodimerizes in yeast, and it has strong transcription activation activity in yeast. These data suggest that, like other bHLH proteins, bHLH93 may function as a transcriptional regulator in the nucleus controlling gene expression. We have identified floral repressor *MAF5* as a major target of bHLH93 to promote flowering in SD. bHLH93 binds to

MAF5 promoter element *in vivo* and in vitro. Other than *MAF5*, *FLC* and *MAF1-2* are also up-regulated in *bhlh93* but at a lower level than *MAF5*. The activation of multiple floral repressors correlates with *bhlh93* flowering phenotype. Taken together, these data suggest that bHLH93 may provide selective advantage for evolution of facultative flowering behavior under varying environmental conditions for reproductive success.

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CHAPTER 1: Literature Review

Plants are sessile organisms and unlike animals they do not move away from unfavorable environmental conditions to ensure optimum growth. Thus, plants have developed versatile genetic networks to withstand the unfavorable conditions and optimize growth. These genetic networks often interconnect to fine-tune plant growth and development. One such sophisticated genetic mechanism in plants is regulation of transition from vegetative to reproductive phase, also known as flowering. Plants sense and respond to their environment to control flowering time. *Arabidopsis thaliana*, a model flowering plant, has been extensively investigated to understand the molecular and genetic basis of flowering time control. These genetic pathways relay environmental and endogenous signals to core flowering time genes, allowing their expression when the time is right for flowering transition. Reproduction at the correct time is very crucial for the survival of the offspring in favorable conditions. Thus, it is very critical for the plant to judge optimum environmental conditions for its reproductive success.

In *Arabidopsis*, along with other factors, four major genetic pathways, namely vernalization, autonomous, photoperiod, GA pathways play central roles in regulating flowering time. These four pathways integrate at downstream genes called floral integrators. Floral integrators, FLOWERING LOCUS T (FT) and SUPPRESSOR OF CONSTANS1 (SOC1) activate expression of floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) which eventually activate stem cells at the shoot apical meristem (SAM) to induce flowering.

VERNALIZATION PATHWAY

Many winter annual and biennial plants flower in spring season after a long exposure to winter, a process called as Vernalization. Few *Arabidopsis* accessions also

have a requirement for vernalization to induce flowering. Plants can differentiate between vernalization and a short exposure to cold in autumn and therefore acquire competence to flower only after several weeks of exposure to cold. Vernalization induces an epigenetic switch of a floral repressor *FLOWERING LOCUS C* (*FLC*), which is active before cold treatment. *FLC* chromatin is repressed in response to a prolonged cold signal and switched to OFF conditions. This 'off switch' overcomes the repression of flowering by FLC and induces the downstream floral integrator genes *FT* and *SOC1*. This epigenetic repression of *FLC* is mitotically stable: i.e. once the vernalization signal is perceived in the SAM and *FLC* is repressed, plants retain their memory of the cold and eventually return *FLC* to the normal active state in the next generation of progeny (Sheldon et al., 2000; Sung et al., 2006; Sheldon et al., 2008; Jiang et al., 2008).

Vernalization-requiring *Arabidopsis* accessions have a dominant allele of the gene *FRIGIDA* (*FRI*). This dominant allele of *FRI* encodes a full-length protein whereas the recessive allele contains mutation that makes the FRI protein non-functional (Johanson et al., 2000). Along with *FRI*, *FLC* is needed for vernalization requirement (Koorneef et al., 1994; Lee et al., 1994). FRI with its family members FRI-LIKE 1 (FRL1) and FRL2 induce *FLC* transcripts to a level to achieve a vernalization requirement (Michaels et al., 2004; Schlappi et al., 2006). FLC, a MADS-box protein, directly bind to and represses the expression of floral integrators *FT*, *SOC1* and *FLOWERING LOCUS D* (*FD*) (Hepworth et al, 2002; Helliwell et al., 2006; Searle et al., 2006). FLC interacts with SHORT VEGETATIVE PHASE (SVP) and together they bind to *FT* and *SOC1* to repress their expression (Fujiwara et al., 2008; Li et al., 2008). FLC belongs to a group of MADS box containing Factors (MAFs) and this MAF clade has *MAF1-5* genes. Among the MAF clade, FLC is the major repressor of flowering, however other *MAF* genes have

been shown to repress flowering as well (Ratcliffe et al., 2001; Ratcliffe et al., 2003; Kim et al, 2010).

First gene reported in vernalization mediated suppression of *FLC* is *VERNALIZATION 2* (*VRN2*) which is a homolog of the *Drosophila* protein SUPRESSSOR OF ZESTE 12 (Chandler et al., 1996; Gendall et al., 2001), which is a part of a Polycomb repression complex (PRC) that modifies chromatin (Muller et al., 2002). Identification of *VRN2* confirmed an epigenetic control of *FLC* chromatin, because in *vrn2* mutants *FLC* expression is reduced during long exposure of cold but the repression is not retained (Gendall et al., 2001). Later it was shown that *FLC* chromatin undergoes two repressive histone modifications, histone H3 Lys 9 (H3K9) and H3K27 methylation, following vernalization (Bastow et al., 2004; Sung and Amasino, 2004). Another vernalization pathway gene, *VERNALIZATION-INSENSITIVE 3* (*VIN3*), is shown to be expressed during long exposure of cold (Sung and Amasino, 2004). In *vin3* mutants, repressive histone modifications such as H3 deacetylation, and H3K9 and H3K27 methylation do not occur on *FLC* chromatin (Sung and Amasino, 2004). VIN3 belongs to a family of PHD finger domain proteins and another member of this family *VERNALIZATION 5* (*VRN5*)/ *VIN3-LIKE 1* (*VIL1*), is also required for epigenetic modification of *FLC* during vernalization (Sung et al., 2006; Greb et al., 2007). Thus, VRN2, VIN3 and VRN5/VIL1 are required for histone modification on FLC chromatin and they appear to form a repressive complex similar to the Polycomb Repressive Complex 2 (PRC2) found in many eukaryotes (Wood et al., 2006; De Lucia et al., 2008). A member of PRC2, ENHANCER OF ZESTE [E(Z)], adds methyl groups at H3K27 residues (H3K27me3) (Muller et al., 2002). *Arabidopsis* has at least two homologs of E(Z) called *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*) in the VIN3 and VIL1/VRN5 complex (Wood et al., 2006; De Lucia et al., 2008). Hence, vernalization process

involves a PRC2-like complex consisting of VRN2, VIN3, VRN5/VIL1, CLF, and SWN in *Arabidopsis* and this PRC2-like complex induces an epigenetic modification at FLC locus.

Thus, the molecular cascades that initiate *FLC* repression during vernalization begin with induction of *VIN3* by cold and enhancement of repressive activity at *FLC* by PRC2-like complex containing VIN3 and VRN5/VIL1 (De Lucia et al., 2008). *VIN3* expression is transient that ceases after cold exposure, but *VRN5/VIL1* is constitutively expressed and remains with *FLC* after cold treatment. (De Lucia et al., 2008). However, following initiation of repressive state of *FLC* by histone modification requires a mechanism to maintain the repressed state through cycles of DNA replication for a mitotically stable modification. Initiation and maintenance of repressive state of *FLC* chromatin is likely to be a progression with no clear delineation (De Lucia et al., 2008; Kim et al., 2009). A plant specific protein, VRN1, and LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)/TERMINAL FLOWER 2 (TFL2) are likely to be involved in maintenance of repression through a feedback loop that maintains H3K9 methylation (Maison and Almouzni, 2004)

A major question that still needs to be explored is how cold is perceived in plants during vernalization. Activation of *VIN3* followed by repression of *FLC* is an output of cold sensing, but what are the factors that sense long exposure of cold is still under investigation. Recent reports have shown a rapid increase in *FLC* antisense transcripts called *COOLAIR* during vernalization, which might activate PRC2 like complex for *FLC* repression (Swiezewski et al., 2009). However there is no direct evidence for a role of antisense transcripts in *FLC* repression during vernalization. More recently a sense transcript called *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) is shown to be necessary for vernalization-mediated repression of *FLC* (Heo and Sung, 2011).

COLDAIR directly interacts with a component of PRC2 complex and targets PRC2 to *FLC* chromatin for epigenetic modification (Heo et al., 2011). However the mechanism by which the expression of *VIN3*, *COOLAIR* and *COLDAIR* are induced by vernalization is still unknown.

AUTONOMOUS PATHWAY

In plants, flowering can occur only after the plant undergoes a transition from juvenile to adult phase (Poething, 2003). This process of control of flowering time comes under the category of autonomous pathway which is independent of environmental cues. In *Arabidopsis*, autonomous pathway has been characterized through mutations in genes that alter flowering time. These mutants have flowering phenotype independent of photoperiod (Simpson and Dean, 2002). Autonomous pathway mutants have been discovered to have a recessive *FRI* allele and therefore, flower without a vernalization treatment. The late flowering autonomous mutant phenotype is due to increased *FLC* expression and vernalization represses *FLC* in autonomous mutants to promote flowering (Michaels and Amasino, 2001).

In *Arabidopsis*, several autonomous pathway genes have been identified, namely *FCA, FLOWERING LOCUS K HOMOLOGY DOMAIN* (*FLK*), *FPA*, and *FY* which are predicted to encode proteins involved in RNA metabolism (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Lim et al., 2004; Manzano et al., 2009). Another autonomous pathway gene *LUMINIDEPENDENCE* (*LD*) encodes a homeodomain-containing protein involved in RNA metabolism and shown to bind RNA (Chan and Struhl, 1997). Other autonomous pathway genes such as *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *FLOWERNG LOCUS D* (*FLD*), MULTICOPY SUPPRESSOR OF IRA1 4 (MSI4)/FVE encode components of chromatin-remodeling

complexes (He, 2009; Kim et al., 2009; Michaels, 2009). REF6 and FLD belong to a class of histone demethylases (Noh et al., 2004; Agger et al., 2008); *FVE* encodes a member of an MSI1-like protein which has been found in several chromatin-modifying complexes in eukaryotes (Ausin et al., 2004; Kim et al., 2004; Henning et al., 2005). Recently small RNAs were proposed to be involved in *FLC* repression by guiding chromatin-modifying complexes to *FLC* chromatin (Baurle and Dean, 2008). Double mutant between *DICER-LIKE1* and *DICER-LIKE2* genes show vernalization-responsive delayed flowering due to *FLC*, similar to autonomous pathway phenotype (Schmitz et al., 2007).

Other than repressing *FLC*, autonomous pathway mutants display pleiotropic effects on growth. This was first noted when Henderson et al (2005) demonstrated that a double mutant between *fy* and *fpa* is lethal . Double mutants between autonomous pathway genes have pleiotropic phenotypes (Veley and Michaels, 2008).

An additional autonomous pathway has been shown to involve the microRNAs, miR156 and miR172 that are expressed independent of day length. miR156 promotes the juvenile phase in *Arabidopsis* and maize to prevent precocious flowering (Wu and Poethig, 2006; Chuck et al., 2007). Oppositely, miR172 promotes flowering by repressing APETALA2-like repressor of *FT* (Aukerman and Sakai, 2003; Jung et al., 2007; Mathieu et al., 2009). miR156 and miR172 show opposite expression patterns where miR156 expression drops during development and miR172 expression increases (Chuck et al., 2007; Wu et al., 2009). These two miRNA form a molecular circuit with *SQUAMOSA PROMOTER BINDING –LIKE* (*SPL*) class of genes, e.g., miRNA156, represses certain *SPL* genes that are positive regulators of miRNA172 expression (Wu et al., 2009). This circuit of regulation controls expression of *FT* and eventually activate floral transition genes, including *SOC1*, *AGAMOUS-LIKE 42* (*AGL42*) and FUL (Wang

et al., 2009a). Thus, autonomous pathway brings an additional level of regulation of flowering time in *Arabidopsis*.

GIBBERELLIC ACID PATHWAY

Arabidopsis require GA for normal growth and it is involved in various developmental switches, including seed germination, juvenile to adult transition, and transition from vegetative to reproductive phases (Mutasa et al. 2008). GA biosynthesis is fine-tuned by GA metabolic genes *(GA 20-OXIDASE* and *GA 3- OXIDASE*) and catabolic genes (*GA 2- OXIDASE*) that maintain a threshold bioactive GA (mainly GA_1 and GA_4) in plants.

Previously it has been shown that GA signaling is mediated by GRAS family of transcription factors called DELLA proteins with conserved amino acid sequence Asp-Glu-Leu-Leu-Ala (D-E-L-L-A). DELLA proteins mainly function as repressor of GA signaling. DELLA family of protein consists of five members namely, REPRESSOR OF GA1-3 (RGA), GA-INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGL2, and RGL3. However, RGA, RGL2, and RGL1 have been shown to repress flowering and fertility (Cheng et al., 2004). The repression of GA signaling by DELLAs is removed by an E3 ubiquitin ligase complex containing SLEEPY1 (SLY1), an F-box protein (Dill et al., 2004). The SCF^{SLY1} complex targets DELLAs to destruction by the ubiquitin-proteasome pathway. Moreover, GA binds to receptor GA-INSENSITIVE DWARF1 (GID1), and this GA-GID1 binding stimulates GID1-DELLA binding (Murase et al, 2008). This molecular cascade is followed by increased affinity of F-box protein SLY1 for DELLAs (Griffiths et al., 2006) and eventual degradation of DELLAs by proteasome mediated pathway. However, there is an additional proteolysis independent GID1 mediated downregulation of DELLA repression (Ariizumi et al., 2008). Thus, GA signaling is

tightly regulated process where GA induces a molecular cascade that involves conformational change in the receptor followed by enhanced binding of an F-box with GA signaling repressors. The GA repressors are finally degraded to promote GA signaling.

In *Arabidopsis*, GA regulates the switch from vegetative to reproductive phase. GA promotes transition of shoot apical meristem to inflorescence meristem that ultimately commits to produce floral meristems. In *Arabidopsis* LD pathway is mainly regulated through CO. However, GA is absolutely required for promotion of flowering under non-inductive SD conditions. This absolute requirement of GA has been shown by *ga1-3* mutant that fail to make bioactive GA and do not flower under SD (Wilson et al., 1992). GA promotes expression of floral integrator *SOC1* (Bonhomme et al., 2000; Moon et al., 2003) and *LFY* (Blazquez et al., 1998) through GAI/RGA DELLA proteins (Gocal et al., 1999, 2001). GA regulates *LFY* through an additional pathway via SOC1. SOC1 interacts with AGAMOUS-LIKE 24 (AGL24) and form an autoregulatory feedback loop (Liu et al., 2008). Thus, GA has a prominent role in promoting flowering time in *Arabidopsis* specifically under SD conditions.

PHOTOPERIOD PATHWAY

Plants respond to day length, called photoperiod, and regulate the flowering time accordingly. The first reports indicating role of photoperiod in flowering were published by Tournois (1914) and Klebs (1918). Garner and Allard (1920) confirmed this report and showed short-day plants (SDPs) flower when the night exceeds a critical length and long day plants (LDPs) flower when day length increases. Day length is perceived in the leaves (Knott, 1934) and flowering occurs in the SAM. This signal, referred to as florigen

(Chailakhyan, 1936) is transmitted to the SAM and the identity of the florigen has been an active field of research.

Arabidopsis is a facultative/quantitative LDP, which means it flowers early in LD and shows delayed flowering in SD conditions (Gregory and Hussey, 1953). Mutants that display delayed flowering as compared to wild type in LD and SD produce more rosette leaves from SAM showing a compromised photoperiod-dependent flowering. On the contrary, hypersensitive mutants flower earlier than wild type and produce fewer leaves. Thus, it is very convenient to identify flowering mutants on the basis of leaf number (Amasino 2010). Photoperiod pathway mutants show a LD or SD specific mutant phenotype, unlike autonomous pathway mutants that have altered flowering phenotype in both SD and LD.

As described earlier, a photoperiod signal called florigen moves from the site of perception in leaves to the site of execution in SAM. FT is now known to be a part of florigen because it is expressed in the leaves and FT protein interacts with a SAM specific protein FD to induce flowering at SAM (Abe et al., 2005). In *Arabidopsis*, FT is shown to move from young leaves to the SAM using FT:GFP and FT:MYC fusion proteins (Corbeisier et al., 2007; Jaeger and Wigge, 2007; Tamaki et al., 2007). This provides strong evidence that FT is an important component of florigen. CONSTANS (CO) and GIGANTEA (GI) induce *FT* expression in the leaves. CO, a zinc finger protein, is a transcription factor and GI, a plant-specific protein, is involved in circadian clock regulation (Fowler et al., 1999; Park et al., 1999). CO is necessary for inducing expression of *FT* and an *FT* relative called *TWIN SISTER OF FT* (*TSF*) (Wigge et al., 2005; Yamaguchi et al., 2005). Following the induction in the leaves, FT acts in the meristem by interacting with FD, a bZIP transcription factor (Abe et al., 2005; Wigge et al., 2005). *CO* is controlled by circadian clock where GI along with FLAVIN-BINDING,

KELCH-REPEAT, FBOX PROTEIN 1 (FKF1) and CYCLING DOF FACTORS (CDFs) regulate *CO* expression. CDFs, which show circadian regulation, bind to *CO* promoter to repress *CO* expression. CDFs in turn are regulated by FKF1 through ubiquitin mediated degradation of CDFs (Imaizumi et al., 2005). GI, a clock protein, physically interacts with FKF1 and stabilizes it (Sawa et al 2007). GI-FKF1 stable complex targets CDFs on *CO* promoter and degrade CDFs to promote *CO* expression (Sawa et al., 2007).

Other than photoperiod, light quality also affects *FT* expression through CO to regulate flowering time in *Arabidopsis*. Blue light receptors, namely CRYPTOCHROME 1 (CRY1) and CRY2 and a red-far red receptor called PHYTOCHROME A (PHYA) are shown to stabilize CO protein, and another red-far red receptor PHYTOCHROME B (PHYB) is shown to promote turnover of CO (Valverde et al., 2004). An E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) and SUPPRESSORS OF PHYA-105 (SPA) family of proteins are involved in CO turnover via proteasome mediated proteolysis (Valverde et al., 2004; Laubinger et al., 2006; Jang et al., 2008; Liu et al., 2008b; Ishikawa et al., 2006). Recently, it has been shown that phloem-specific expression of *GUS-SPA1* reduces FT transcript levels and *SPA1* expression in the phloem is sufficient to inhibit flowering (Ranjan et al., 2011).

Following *FT* activation in leaves and movement through phloem to meristem, FT and FD complex activate expression of *SOC1* and downstream floral-activation genes such as *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) in the meristem (Michaels et al., 2005; Yoo et al., 2005; Abe et al., 2005; Wigge et al., 2005). These downstream floral meristem genes activate flanking groups of cells of the SAM to differentiate into floral meristem. The transition from vegetative to reproductive phase in *Arabidopsis* is an irreversible process because of multiple positive feedback loops by genes expressed in the SAM, such

as *AP1*, *LFY*, *SOC1*, and *AGAMOUS-LIKE 24* (Liljegren et al., 1999; Michaels et al., 2005; Sablowski 2007; Lee et al., 2008; Liu et al., 2008a).

To avoid precocious flowering by photoperiod induction, a floral repression mechanism involves two TEMPRANILLO genes (TEM1 and TEM2). TEM1 and TEM2 directly repress *FT* expression (Castillejo et al., 2008). However, TEMs are downregulated over development to mark the timing of flowering. Recently a miR172 was reported to be involved in flowering time regulation and another group of AP2 domain-containing genes *TARGET OF EAT 1-3* (*TOE1-TOE3*) have a miR172 target site (Aukerman and Sakai, 2003; Chen, 2004; Park et al., 2002; Jung et al., 2007). Also, in the meristem *FT* is counteracted by a closely related gene called TERMINAL FLOWER 1 (TFL1) (Ahn et al., 2006; Kardailsky et al., 1999, Kardailsky et al., 1999). These findings suggest that photoperiod pathway presents a robust regulation of flowering via several floral activators and repressors.

CONCLUSION

Flowering in plants is a tightly regulated process where floral activators and repressors act at several levels. Reproductive success of plants mainly depends on time of flowering under optimal conditions. Untimely flowering may lead to inferior progeny seeds that will not be able to withstand unfavorable conditions and will eventually affect seed yield. Flowering in *Arabidopsis* is synchronized by four major genetic pathways named vernalization, autonomous, GA, and photoperiod pathways. These pathways consist of several floral repressors and activators. However, the final product of regulation by floral repressors and activators is activation of floral-identity genes under favorable conditions only.

Among the repressors, MADS-domain and AP2-domain proteins play the major roles in repressing flowering time in *Arabidopsis*. The most studied floral repressor MADS-domain transcription factor (MAF) *FLC* acts in both leaves and SAM to integrate the vernalization and autonomous pathways (Schmitz et al., 2007; Simpson et al., 2004). FLC is regulated at both epigenetic and transcriptional levels by several factors (Farrona et al., 2008; Schmitz et al., 2007). FLC has been shown to directly bind *FT* and *SOC1* to repress their expression (Helliwell et al., 2006; Searle et al., 2006). FLC belongs to MAF family, which has other MAF genes such as *MAF1-5* that has been shown to act as floral repressors as well (Ratcliffe et al., 2001; Ratcliffe et al., 2003; Kim et al, 2010). Other floral repressors such as AP2 domain-containing proteins TEM1 and TEM2 directly repress *FT* expression and thus, quantitative balance between the activator CO and the repressor TEMs determines FT levels in LD pathway. Over the development, TEMs are downregulated to bring CO levels up in the balance. The higher CO levels activate *FT* to induce flowering in photoperiod pathway. Additional floral repressor such as miR172 and TFL1 regulate *TOE1-TOE3* and *FT* respectively (Aukerman and Sakai, 2003; Chen, 2004; Park et al., 2002; Jung et al., 2007; Ahn et al., 2006; Kardailsky et al., 1999, Kardailsky et al., 1999). Thus, floral repressors are essential to inhibit flowering under unfavorable conditions.

Among the floral activatorrs, CO plays a major role under LD pathway to induce downstream floral integrators *FT* and *SOC1*. CO levels are regulated by CDF1, FKF1 and GI complex. However, in vernalization, repression by FLC is overcome by cold signal that activates *COLDAIR* intronic RNA and *VIN3*. *FLC* repression leads to activation of *FT* and *SOC1*. Autonomous pathway also involves repression of *FLC* via several autonomous pathway genes such as *FCA, FPA, FY, FLK* among others. Recently microRNAs are shown to regulate flowering time under autonomous pathway.

Phytohormones especially GA brings additional positive regulation in flowering time, specifically under SD. GA directly affects downstream floral integrator *SOC1* and *LFY*. Therefore, floral activators negate the effect of floral repressors to optimize time of flowering.

In summary, flowering is a crucial transition in growth pattern in plants and involves a complex network of genes. These genes interact at various levels to induce flowering only under optimum conditions. The correct timing of flowering ensures reproductive success in plants.

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CHAPTER 2: Regulation of flowering time by a bHLH transcription factor in *Arabidopsis*

ABSTRACT

Flowering in plants is a dynamic and synchronized process where various cues including age, day-length, temperature and endogenous hormones fine-tune the timing of flowering for reproductive success. *Arabidopsis thaliana* is a facultative long day (LD) plant where LD photoperiod promotes flowering. *Arabidopsis* still flowers under short-day (SD) conditions, albeit much later than LD conditions. Although, factors regulating the inductive LD pathway have been extensively investigated, the non-inductive SD pathway is much less understood. Here we identified a critical transcription factor called bHLH93 (basic Helix-Loop-Helix 93) that is essential to induce flowering specifically under SD conditions in *Arabidopsis*. *bhlh93* mutants do not flower under SD conditions, but flowers similar to wild type under LD conditions. The late flowering phenotype is rescued by exogenous application of GA and prolonged vernalization, suggesting that bHLH93 acts in parallel with the GA and vernalization pathways to promote flowering. *bHLH93* is expressed in meristematic regions and its expression peaks at 8 hours after dawn under SD conditions. bHLH93 is also localized to the nucleus. Taken together, these data suggest that bHLH93 is a key transcription factor necessary for promotion of flowering under noninductive SD conditions. bHLH93 may provide selective advantage for evolution of facultative plants under varying environmental conditions.

INTRODUCTION

Flowering, a transition from vegetative to reproductive phase, is one of the critical developmental transitions in plant life cycle. The time of flowering in plants is synchronized by various endogenous and environmental cues to produce flowers only under optimal conditions. Flowering time in the model plant *Arabidopsis thaliana* has been extensively studied. Four major genetic pathways, namely, vernalization (long exposure to cold), autonomous (genetic makeup), hormones, and photoperiod (day-length) pathways regulate flowering time in *Arabidopsis*. These four genetic pathways emerged to control the expression of floral integrator genes, such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) that activate the downstream floral identity genes (e.g., *APETALA1, AP1* and *LEAFY, LFY*) to promote flowering (Michaels and Amasino, 1999; Blazquez and Weigel, 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000; Moon et al., 2003; Moon et al., 2005).

Vernalization pathway controls flowering time through the floral repressors *FLC* and *FLC*-clade members (Kim et al., 2009). The FLC-clade consists of MADS-box transcription factors *FLOWERING LOCUS M / MADS AFFECTING FACTOR 1* (*MAF1*) and *MAF 2-5* (Kim and Sung, 2010). Winter annual accessions of *Arabidopsis* containing *FRIGIDA* (*FRI*) and *FLC* require vernalization treatment to overcome repression of flowering (Michaels and Amasino, 1999). In non-vernalized plants, FLC represses expression of *FT* and *SOC1* in phloem and in the meristem and *FD* in the meristem (Searle et al., 2006). After vernalization treatment, *FLC* expression is strongly repressed by epigenetic regulation (Kim et al., 2009). Repression of *FLC* leads to activation of downstream floral integrators, *FT* and *SOC1* that allow plants to flower after a long

duration of cold exposure. Autonomous pathway also represses *FLC* through *LD*, *FCA*, *FY*, *FPA*, *FLD*, *FVE*, *FLK*, and *REF6* (Noh et al., 2004; Simpson, 2004). *FCA, FY, FPA*, and *FLK* proteins are predicted to be involved in RNA metabolism (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Lim et al., 2004). FCA and FPA are RNA-binding proteins involved in repression of *FLC* and other genes. FY interacts with FCA's WW domain to promote flowering (Simpson et al., 2003). FVE, FLD, and REF6 have domains similar to chromatin-modifying components, and *FLD* and *REF6* are predicted to encode histone demethylases (He et al., 2003; Noh et al., 2004; Jiang et al., 2007). In summary, both vernalization and autonomous pathways converge on FLC, which regulates downstream floral integrator genes to regulate flowering time.

Several phytohormones, such as GA, brassinosteroid (BR), nitric oxide (NO) and salicylic acid (SA) crosstalk to fine-tune the timing of flowering in *Arabidopsis* (Davis, 2009). Among all the hormones, the roles of GA in controlling flowering time have been best understood. Under non-inductive short day (SD) photoperiod, *ga requiring 1 (ga1)* mutant fails to flower suggesting an absolute requirement of GA signaling in SD conditions (Wilson et al., 1992). GA directly promotes *SOC1* and *LFY* expression under SD conditions (Moon et al., 2003; Mutasa-Göttgens and Hedden, 2009). Increased SOC1 in turn activates downstream floral meristem identity genes, *LFY* and *AP1*, to promote flowering. This relay of information from GA to SOC1 occurs through degradation of the DELLA proteins RGA and RGL2 with a partial contribution from RGL1 (Cheng et al., 2004).

Photoperiod (day-length) plays a very critical role in controlling flowering time in *Arabidopsis* (Fornara et al., 2010). The photoperiod signal is perceived in the leaves and this signal, often called florigen, moves to the shoot apical meristem (SAM) where flowers are produced. *Arabidopsis* is a facultative long day (LD, 16h light/8h dark) plant

where long day acts as an inductive photoperiod to promote flowering, and flowering is delayed under non-inductive SD (8h light/16h dark) photoperiod. The biochemical basis for difference in flowering time under LD and SD is very well documented through an external coincidence model (Yanovsky and Kay, 2003). According to this model, light plays two major roles: resetting the circadian clock that generates daily oscillation of *CO* and regulating CO protein stability (Imaizumi and Kay, 2006). The daily oscillation of *CO* is regulated in part by two antagonistic groups of genes: activators *FLAVIN-BINDING, KELCH REPEAT, AND F-BOX 1(FKF1), GIGANTEA (GI),* and repressors *ELF3, CYCLING DOF FACTOR1 (CDF1),* and *RED AND FAR-RED INSENSITIVE 2 (RFI2)* (Fowler et al., 1999; Nelson et al., 2000; Covington et al., 2001; Suárez-López et al., 2001; Imaizumi et al., 2005; Chen and Ni, 2006). In the dark, CDF1 is present at the *CO* promoter repressing *CO* expression. However, after light is perceived in the leaves, GI interacts with the F-box protein FKF1 and the GI-FKF1 complex degrades CDF1 through ubiquitin-mediated proteolysis. This leads to de-repression of *CO* transcription. On the other hand, CO stability is regulated posttranslationally, where photoreceptors such as phytochrome A (phyA*),* cryptochromes *(*cry1-2*)* have been shown to prevent CO protein degradation, while phyB promotes CO degradation (Valverde et al., 2004). Thus, CO mRNA and protein level peaks at 12h after dawn, which coincides with light in LD, but dark in SD. Therefore, increased CO protein promotes expression of *FT* only under LD. FT moves through phloem to meristem, where it associates with FD, and FT-FD complex activates expression of *SOC1* and downstream floral identity genes such as *AP1* and *LFY* to promote flowering (Abe et al., 2005; Michaels et al., 2005; Wigge et al., 2005; Yoo et al., 2005). However, in SD, CO protein peaks in the dark, where it is degraded through the COP1-SPA complex and thus, shows a delayed flowering response.

Although the LD photoperiod pathway is well known, recent evidence suggests the presence of a non-inductive SD pathway for promotion of flowering time. For example, Plant Homeo Domain finger-containing proteins such as VIN3-LIKE 1/VRN5 and 2 (VIL1-2) have been shown to promote flowering through epigenetic repression of *MAF1* and *MAF5* genes respectively under SD conditions (Sung et al., 2006; Kim and Sung, 2010). *vil1* and *vil2* mutants flower later only under SD conditions, but eventually flower. On the other hand, *spa1, cop1* and *cul4cs* mutants display early flowering only under SD conditions (Mcnellis et al., 1994; Ishikawa et al., 2006; Laubinger et al., 2006; Jang et al., 2008; Chen et al., 2010; Ranjan et al., 2011). SPA1 physically interacts with COP1, and COP1-SPA1 associates with CUL4 forming an E3 ubiquitin ligase, which promotes CO degradation through ubiquitin-mediated proteolysis (Laubinger et al., 2006; Jang et al., 2008; Chen et al., 2010). It appears that these factors are regulating components that control flowering time typically under LD pathway. Nonetheless, the non-inductive SD pathway is still poorly understood. In addition, the factors necessary for plants such as *Arabidopsis thaliana* to evolve as a facultative LD plant is still unknown. Here we describe a basic-helix-loop-helix (bHLH) transcription factor, bHLH93, which functions as a critical component of the SD photoperiod pathway. *bhlh93* mutants fail to flower only under SD conditions, but flower like wild type under LD conditions. Therefore, bHLH93 may represent a pivotal transcription factor necessary for *Arabidopsis thaliana* to evolve as a facultative LD plant.

MATERIALS AND METHODS

Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24-h light or long day (LD, 16h light, 120 $\mu molm^{-2}s^{-1}$ and 8h dark), or short day

(SD, 8h light, 200 µmol$m^{-2}s^{-1}$ and 16h dark), or 14hL/10hD or 12hL/12hD or 10hL/14hD photoperiod at 21^0C $\pm$ 0.5^0C. Light fluence rates were measured using a spectroradiometer (model EPP2000; StellarNet Inc., Tampa, FL) as described (Shen et al., 2005). T-DNA-tagged *bhlh93* seeds from SALK collection were obtained from the *Arabidopsis* Biological Resource Center (Alonso et al., 2003). Seeds were surface sterilized and plated on Murashige and Skoog growth medium (GM) containing 0.9% agar without Suc (GM - Suc) as described (Shen et al., 2005). After 4 days of stratification at 4^0C, seeds were exposed to SD or LD or continuous white light conditions. T-DNA insertion lines were PCR-screened using primers described in table 2.1.

Complementation analysis

To confirm the role of *bHLH93* in controlling flowering time in SD, a genomic DNA fragment containing the entire *bHLH93* gene with 1953 bb promoter and 531 bp 3'-untranslated region (*pbHLH93:bHLH93)* was transformed into *bhlh93-1* background. Single-locus transgenic plants were selected based on Kanamycin resistance. Homozygous transgenic lines were grown in SD and flowering time was quantified using number of days and number of rosette leaves.

Exogenous GA application and vernalization response assays

One hundred µM GA_4 was applied twice a week directly on the meristem of the Wt, *bhlh93-1, bhlh93-2, ga1* and *phyB* mutant plants grown under SD starting at day 6 until the plants flowered (increasing amount of GA_4 starting from 10 µl to 300 µl). Flowering time was quantified using the number of rosette leaves produced at the time of bolting. For vernalization response assays, seeds were surface sterilized and plated on GM – Suc plates as described above and exposed to SD for germination for 7 days. Then

the seedlings were transferred to 4^0C for 8 weeks. Seedlings were transplanted on soil and then grown under SD (8h light/16h dark) conditions at 21^0C until bolting.

Spatial and temporal analyses of *bHLH93* expression

For tissue-specific and developmental expression of *bHLH93*, a 3680 bp DNA fragment including the 1953 bp promoter and the complete open reading frame without the stop codon was amplified by PCR using PFU polymerase (Stratagene, La Jolla, CA) and cloned into pBI121 vector to replace the 35S promoter. This construct (*pbHLH93:bHLH93-GUS*) was then transformed into wild type using the *Agrobacterium*-mediated transformation protocol as described (Clough and Bent, 1998). Single-locus transgenic plants were selected based on Kanamycin resistance. A transgenic plant carrying the *pbHLH93:bHLH93-GUS* transgene in wt background was crossed into *bhlh93-1* mutant and homozygous lines were produced for GUS analysis. Homozygous transgenic lines were grown on GM-Suc plates for various time points under SD, LD and continuous light as indicated, and histochemical GUS assays were performed as described (Shen et al., 2007).

Subcellular localization of bHLH93

For subcellular localization assay, the open reading frame of *bHLH93* without the stop codon was cloned into pENTR_D_TOPO vector (Invitrogen Inc., Carlsbad, CA) and recombined with a destination vector pB7WGY2 (Karimi et al., 2005). This construct, named p35S:*bHLH93-YFP*, was sequenced and then transformed into wild type plants using the *Agrobacterium*-mediated transformation protocol as described (Clough and Bent, 1998). Several homozygous transgenic plants containing the transgene were identified based on Basta selection. Four-day-old dark-grown p35S:*bHLH93-YFP*

seedlings were used to investigate the sub-cellular localization of bHLH93 in stable transgenic plants using a fluorescent microscope.

Semi-quantitative RT-PCR analyses

Total RNA was isolated from 10 day-old *bhlh93-1* mutant and wild-type Col-0 seedlings grown under SD using the RNeasy plant mini kit (Qiagen, Valencia, CA). For reverse transcription reaction, total RNA was treated with DNase I to remove genomic DNA. One µg of total RNA was reverse transcribed using the RT-PCR kit (Invitrogen Inc., Carlsbad, CA), and first-strand cDNA was used as a template for PCR amplification. For semi-quantitative RT-PCR, 20 µL cDNA was diluted to 40 µL with water and 1 µL of diluted cDNA was used for PCR amplification of *MAF5, SOC1, CO, FT, ELF7, EFS, FLC, AGL15, AGL24, AGL18, AGL19, SVP, VIL1, VIL2, GAI, RGA, GID1a, GID1b* and *UBQ10* fragments using gene-specific primers. The UBQ10 fragment was used as a control to normalize the amount of cDNA used. The RT-PCR primer sets are shown in Supplemental Table S1. For Southern blots, *CO* and *FT* probes were labeled using the random primer-labeling kit (TaKaRa, Berkeley, CA). *CO* and *FT* were amplified using semi-quantitative RT-PCR using 24 and 25 cycles, respectively. Amplified products were separated on a 1% agarose gel and blotted onto membrane for Southern hybridization using the labeled *CO* and *FT* probes. Blots were washed for 15 min at low stringency followed by a high-stringency wash at 42^0C, and then the membranes were exposed to a phosphor screen (Kodak, Rochester, NY) at room temperature for overnight. The phosphor screen was developed using the Molecular Imager FX system (Bio-Rad Laboratories, Inc., Hercules, CA).

Genome wide microarray analyses

For microarray, total RNA was isolated from 10-day-old *bhlh93-1* and wild-type Col-0 seedlings grown under SD using the RNeasy plant mini kit (Qiagen, Valencia, CA). Five µg total RNA from Wt and *bhlh93* mutants was reverse transcribed using the RT-PCR kit (Invitrogen Inc., Carlsbad, CA). Microarray hybridization experiment was performed according to user manual of Roche NimbleGen *Arabidopsis thaliana* 12x135K Array (090717 Athal TAIR9 exp HX12; Cat No. 05543746001) (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Log scale gene expression values were calculated using a robust multiarray analysis (RMA). Fold change values between various genotypes and treatments were calculated using the mean expression value of the three biological replicate samples. Statistically significant differential expression by two-fold (SSTF) with a false discovery rate (FDR) of 3.5% was determined using the SAM packages (http://www-stat.stanford.edu/~tibs/SAM/) (Tusher et al., 2001). SSTF genes were defined as those that differ by ≥2-fold with q values ≤ 0.05.

To simplify the functional classification analysis, a single functional category was assigned to each locus as indicated. Functional designations for each locus were determined using a recent annotation of the *Arabidopsis* genome (TAIR10) as well as Gene Ontology (GO) information. Any gene product targeted to the chloroplast was assigned to the Photosynthesis/Chloroplast category. Gene products with predicted or established transcription or DNA binding activity were assigned to the Transcription category.

RESULTS

Isolation and characterization of *bhlh93* mutant

During genome-wide analyses of T-DNA insertion lines for *Arabidopsis bHLH* transcription factor genes (Toledo-Ortiz et al., 2003), we have identified two independent alleles of homozygous T-DNA insertion mutants in the *bHLH93* (Fig. 2.1A). Both alleles have T-DNA insertions in the first exon of the *bHLH93* gene. To investigate whether *bHLH93* is expressed in these mutant lines, we performed a semi-quantitative RT-PCR analysis on Wt and *bhlh93* mutant alleles. Both alleles did not show any detectable expression of *bHLH93* compared to wt, suggesting that they are null mutants (Fig 2.1B). During growth of these mutants and wt plants under continuous light conditions in a growth room, we observed strong visible defects in the mutant plants compared to wt plants. *bhlh93* mutants displayed shorter stature, curly and darker green leaves, and reduced fertility compared to wt plants (Fig. 2.1C; Fig. 2.2). Measurement of internode lengths showed that internode #1, 3 and 4 are significantly shorter than that of wt plants (Fig. 2.2A). These data suggest that bHLH93 regulates plant stature, chlorophyll content and fertility.

bHLH93 regulates flowering time specifically under short day conditions

To investigate adult phenotypes of *bhlh93* mutant lines, we grew wt and *bhlh93* in different photoperiod conditions. Under LD (16h light/8h dark) conditions, *bhlh93* mutants flowered similar to wt plants (Fig. 2.3A). However, under SD (8h light/16h dark) conditions, *bhlh93* mutants failed to flower from the primary meristem (Fig 2.3B). Although we observed occasional bolting from auxiliary meristem at a low frequency (10-20%), most plants undergo senescence without flowering from the primary meristem. We quantified the flowering phenotype using both the number of rosette leaves formed at

the time of flowering and days taken to flower. Results showed that the number of rosette leaves and days to flower were similar for both mutant and wt plants grown under LD conditions (Fig. 2.3C, E), while *bhlh93* mutant failed to flower even after producing ~100 leaves under SD conditions (Fig. 2.3D, E). These data suggest that bHLH93 regulates flowering time specifically under SD conditions.

bHLH93 can complement the *bhlh93* mutant phenotypes

Although two independent T-DNA insertion alleles of *bhlh93* mutant displayed the late flowering phenotype under SD conditions, we transformed *pbHLH93:bHLH93* transgene with 2 kb promoter along with the entire coding region into *bhlh93-1* mutant background for complementation analyses. We selected independent transgenic plants and examined their flowering time phenotype. Results showed that the native *bHLH93* gene rescued the *bhlh93* mutant phenotype under SD (Fig 2.4A, B). These data confirmed that the mutant phenotype was indeed due to a disruption in the *bHLH93* gene.

bhlh93 is a recessive mutant, as the heterozygous plants flowered like wt plants (data not shown). The heterozygous plants also did not display the other morphological phenotypes including the short stature, curly and darker leaves, and reduced fertility. Ectopic expression of *bHLH93* (*p35S:bHLH93-LUC* and *pbHLH93:bHLH93-GUS*) did not result in any observable difference compared to wt, including flowering time under either SD or LD conditions (Fig. 2.5, data not shown). The failure to flower only under SD suggests that *bHLH93* is essential to induce flowering under SD.

Day-length is critical for bHLH93 function

Because *bhlh93* mutant never flowered under SD, but flowered similar to wt under LD conditions, we examined the requirement of different lengths of daytime for *bhlh93* to flower. We grew wt and *bhlh93* under 16hL:8hD (LD), 14hL:10hD,

12hL:12hD, 10hL:14hD, 8hL:16hD (SD) conditions. Wt plants displayed late flowering phenotype under 14hL:10hD photoperiod conditions compared to LD (16hL:8hD) conditions (Fig. 2.6). However, wt plants still flowered earlier than SD (8hL:16hD) conditions. In addition, the flowering time for wt plants was similar under 12hL:12hD, 10hL:14hD, 8hL:16hD (SD) conditions, suggesting that the LD photoperiod pathway is not functional when the day-length is shortened to 12h light conditions. Under 14hL:10hD conditions, *bhlh93* mutants flowered; however, they displayed significantly later flowering compared to wt plants. Strikingly, *bhlh93* mutants failed to flower when the LD photoperiod pathway is turned off. These data suggest that the late flowering phenotype of *bhlh93* mutant is strictly SD specific.

Exogenous GA_4 application rescues the late flowering phenotype of *bhlh93* mutant under SD conditions

Gibberellic acid (GA) has been shown to be essential to promote flowering predominantly under SD conditions (Wilson et al., 1992). To examine whether the flowering phenotype of *bhlh93* mutant can be rescued by exogenous application of GA, we externally applied biologically active GA_4 on the meristem of Wt, *bhlh93* and *phyB* mutants grown under SD conditions. The *bhlh93* mutant flowered same as Wt under SD conditions with exogenous GA application, suggesting that GA rescues the mutant phenotype under SD (Fig. 2.7; Fig. 2.8A). Interestingly, exogenous GA application partially rescued the other phenotypes such as curly and twisted leaves of *bhlh93* under SD conditions (Fig. 2.8B). These data suggest that either *bHLH93* is involved in GA biosynthesis and/or signaling or GA is acting further downstream from bHLH93 in regulating flowering time under SD conditions.

To examine whether the expression of GA signaling genes (*GID1a, GID1b, GAI* and *RGA*) or GA regulated floral integrator gene (e.g., *SOC1*) are affected in the *bhlh93*

mutant relative to wt plants, we performed semi-quantitative RT-PCR analyses for these selected genes. Results showed that the expressions of these genes in *bhlh93* mutant seedlings are largely similar to wt seedlings (Fig. 2.9A, C, see below), suggesting that GA is acting further downstream from bHLH93 in regulating flowering time.

Prolonged vernalization treatment rescued the late flowering phenotype of *bhlh93* mutant under SD conditions

To investigate whether *bhlh93* is defective in vernalization pathway, we vernalized wt and *bhlh93* mutant plants for 8 weeks at 4^0C and then transferred to SD conditions to examine flowering time. Vernalization results in flowering of *bhlh93* mutants similar to wt plants (Fig. 2.10). Semi-quantitative RT-PCR and microarray analyses of selected vernalization pathway genes also did not show any difference in expression between non-vernalized wt and *bhlh93* mutant (Fig. 2.9B, see below). These data suggest that the vernalization pathway is not defective in *bhlh93* mutant, and bHLH93 may act in parallel with vernalization pathway to regulate flowering time.

bhlh93* is epistatic to *phyB*, *rga* and *pif1

Because the flowering phenotype of *bhlh93* is rescued by GA application, we examined the epistatic interaction between *bhlh93* and GA signaling mutants. *phyB* flowers early under both SD and LD conditions, and phyB and PIF1 are also known to regulate GA biosynthesis/signaling pathways. We created double mutants between *bhlh93* and *phyB*, *rga* and *pif1*, and investigated their flowering time under SD conditions. Strikingly, all the double mutants failed to flower similar to *bhlh93* single mutant under SD conditions (Fig. 2.11), suggesting that *bhlh93* is epistatic to all these mutants. These data also suggest that bHLH93 functions independent of known components of the SD pathway.

***bHLH93* is expressed in a tissue-specific and developmental stage dependent manner**

To investigate the spatial and temporal expression patterns of *bHLH93* under SD, LD and continuous light conditions, we produced transgenic plants expressing bHLH93 fused to GUS (β-Glucouronidase) from the native *bHLH93* promoter (*pbHLH93:bHLH93-GUS).* The construct was transformed into Wt background and homozygous single copy transgenic lines were selected. We performed histochemical GUS assays at different stages of development (2, 4, 6, 8 and 10 day-old seedlings) using *pbHLH93:bHLH93-GUS* plants grown under different photoperiods. Under SD conditions, these seedlings displayed GUS activity mainly in root tips and SAM with weak expression in cotyledons throughout the development (Fig. 2.12A-E). GUS activity was observed in the hypocotyl only at day 2 with reduced or no activity at older ages. Under LD conditions, strong GUS activity was observed in the hypocotyl at day 2 and in root tips and SAM throughout the developmental stages (Fig. 2.12F-J). The GUS activity was reduced from cotyledons during developmental stage with strongest activity in two-day old seedlings (Fig. 2.12F) and almost no activity in 10-day old seedlings (Fig. 2.12J) under LD conditions. However, 8 and 10 day-old seedlings displayed strong GUS activity in the primary leaves (Fig. 2.12H-J). The expression pattern in the hypocotyls is similar under both SD and LD with strongest activity at day 2 and a gradual reduction in activity from day 4 to 10 during development (Fig. 2.12A-J). On the other hand, *bHLH93-GUS* shows a very unique expression pattern under continuous light (Fig. 2.12K-O). Strong GUS activity was observed in the root tips throughout developmental stages (Fig. 2.12K-O) and in SAM from 6-day old seedlings (Fig. 2.12M) under continuous light. Surprisingly, very faint or no GUS activity was observed in the cotyledons under this condition (Fig. 2.12K-O). A common feature among SD, LD and continuous light-grown plants is the strong GUS expression in the root tips (A-Q) and veins of leaves (Fig.

2.12A-R). These data are largely consistent with digital expression data on publicly available web sites (Fig. 2.13), and suggest that *bHLH93* is expressed in a tissue specific and developmental stage-dependent manner and may function in specific tissues and/or in specific developmental stages.

The expression pattern of *bHLH93* was monitored during diurnal growth conditions using publicly available data (http://diurnal.cgrb.oregonstate.edu/diurnal_details.html) (Michael et al., 2008). These data showed that *bHLH93* expression is regulated under diurnal conditions with a peak at 12-16 h after dawn under SD conditions (Fig. 2.14A). Under LD conditions, *bHLH93* is expressed constitutively without much variation (Fig. 2.14B). To confirm these data, we performed semi-quantitative RT-PCR in Wt and *phyB* mutant background grown under SD conditions. We examined *bHLH93* expression in *phyB* because *phyB* mutants flower earlier than Wt under SD conditions, a phenotype opposite of *bhlh93*. We observed a peak of expression of *bHLH93* gene at 12h after dawn in Wt as compared to a peak at 16h after dawn in *phyB* mutant (Fig. 2.15). These data are largely consistent with previous reports (Fig. 2.14) (Michael et al., 2008), and suggest that a more robust diurnal regulation of *bHLH93* expression observed under SD conditions might be important for its role in regulation of flowering time specifically under SD conditions.

bHLH93 is localized to the nucleus

To determine the subcellular localization of bHLH93, we introduced *p35S:bHLH93-YFP* transgene into Wt background and selected single copy homozygous transgenic plants. These plants expressing bHLH93-YFP fusion protein were stained with DAPI and examined under fluorescence microscope. Strong YFP fluorescence was observed in an organelle that was also stained with DAPI (Fig. 2.12S), suggesting that

bHLH93-YFP is localized in the nucleus in these stable transgenic plants. These data suggest that bHLH93 is a nuclear protein. We also examined whether bHLH93 can homodimerize using yeast two-hybrid assays. Results showed that bHLH93 has strong transcription activation activity in yeast. Moreover, bHLH93 can homodimerize in yeast two-hybrid assays (Fig. 2.16), suggesting that bHLH93 might function as a transcriptional regulator in the nucleus controlling gene expression as expected like other bHLH proteins.

bHLH93 modestly controls several genes implicated in regulating flowering time

Because *bhlh93* displayed such strong flowering time phenotype, we investigated the molecular phenotype of *bhlh93* mutant compared to wt control. We performed semi-quantitative RT-PCR analyses for flowering time genes (e.g., *SOC1, ELF7, EFS, AGL15, AGL24, AGL18, AGL19, SVP, CO* and *FT*) using RNA isolated from wt and *bhlh93* mutant grown under SD conditions. Results showed that none of the selected genes are differentially expressed between wt and *bhlh93* mutant (Fig. 2.9C, D), suggesting that bHLH93 may regulate novel genes involved in flowering time specifically under SD conditions.

To identify differentially expressed genes between wt and *bhlh93*, we performed a genome wide expression profiling using 10 day-old Wt and *bhlh93* mutant seedlings grown under SD conditions. We harvested triplicate independent biological samples at the end of the dark period and 4h after the light is turned on, and used NimbleGen chip to perform microarray experiment. Data analyses showed that expression of 821 genes change statistically significantly by twofold (SSTF) in *bhlh93* relative to Wt (Table 2.2). Out of these SSTF genes, 35 and 122 genes were induced in the dark and light, respectively, with only two genes induced under both dark and light conditions. For

repressed gene category, 45 and 625 genes were repressed in the dark and light, respectively, with only three genes repressed under both dark and light conditions (Fig. 2.17A). These data suggest that bHLH93 regulates expression of largely distinct set of genes in the dark and light conditions to promote flowering time.

Functional categorization for these SSTF genes was performed using GO terms. A large proportion of induced and repressed genes under both dark and light conditions were unknown genes (Fig. 2.17B). A high percentage of repressed genes under both dark and light also includes the "other" category. Strikingly, 48% of the light repressed genes belong to the transposable element genes (Fig. 2.17B). The significance of differential regulation of these large numbers of "unknown", "other" and "transposable element" genes in *bhlh93* mutant is unknown.

Although microarray analyses did not uncover any known target gene that can explain the dramatic late flowering phenotype of the *bhlh93* mutant, a closer look at the differentially regulated genes revealed a set of genes that may play cumulative roles in regulating flowering time under SD conditions (Fig. 2.17C). Five genes involved in repression of flowering time (e.g., *NIA2, PGI1, SKB1* and *TCTP*) (Yu et al., 2000; Wang et al., 2007; Berkowitz et al., 2008; Seligman et al., 2008) or GA signaling (e.g., *GASA4*) (Rubinovich and Weiss, 2010) are downregulated in *bhlh93* mutant compared to wt controls under both dark and light conditions (Fig. 2.17C). Similarly, three genes (e.g., *AGF1, CYP714A2* and *NF-YC9*) involved in GA metabolism/signaling and/or repression of flowering time are induced in *bhlh93* mutant compared to wt controls (Matsushita et al., 2007; Kumimoto et al., 2010; Zhang et al., 2011). Therefore, the net result of upregulation of multiple floral repressors and down regulation of multiple floral activator genes may contribute to the strong flowering time phenotype of the *bhlh93* mutant. Alternatively, the differential regulation of large numbers of "unknown", "other" and

"transposable element" genes in *bhlh93* mutant may contribute to its strong late flowering phenotype under SD conditions.

DISCUSSION

Arabidopsis thaliana is a facultative LD plant where transition from vegetative to reproductive phase is accelerated under LD conditions compared to SD conditions. *Arabidopsis* still flowers under SD conditions, but much later than LD conditions. The molecular basis for the facultative flowering behavior is still unknown. Here we provide genetic evidence that a critical transcription factor, called bHLH93, is essential to induce flowering specifically under SD conditions in *Arabidopsis*. Some autonomous pathway mutants also fail to flower under SD. However, they flower very late under LD as well (Kim et al., 2009). Unlike known late-flowering mutants, *bhlh93* mutants failed to flower only under SD conditions, but not under LD conditions (Fig. 2.3). Thus bHLH93 is absolutely required for the transition from vegetative to reproductive phase under SD conditions.

Previously, flowering time mutants affected only under SD conditions have been reported. For example, *phyB*, *dnf*, *spa1* and *cop1* flower early whereas *vil1* and *vil2* flower late specifically under SD conditions (Reed et al., 1993; Mcnellis et al., 1994; Ishikawa et al., 2006; Laubinger et al., 2006; Sung et al., 2006; Kim and Sung, 2010; Morris et al., 2010). However, *vil1* and *vil2* mutants display quantitative difference in flowering time compared to wild type and still flower under SD conditions (Sung et al., 2006; Kim and Sung, 2010). Although *ga1* mutants also failed to flower under SD, *ga1* flowers significantly later under LD as well (Wilson et al., 1992). However, *bhlh93* mutants fail to flower in SD without any discernible delay in flowering under LD

conditions. Therefore, bHLH93 represents a novel positive regulator of floral transition functioning specifically under SD conditions.

Phenotypic characterizations showed that flowering time defect in *bhlh93* mutants is independent of GA and vernalization pathways (Figs. 2.7, 2.8, 2.10). On one hand, the stunted growth, reduced fertility, curly and darker leaves suggest that *bhlh93* may have defects in GA pathway. In support of this hypothesis, exogenous GA application completely rescued all these phenotypes including the flowering time phenotype (Fig. 2.8). However, several lines of evidence suggest against this possibility. First, curly and darker leaves are characteristics for the majority of the late flowering mutants and are not specific to *bhlh93* mutants. Second, unlike GA deficient mutants like *ga1*, the rosette size of the *bhlh93* mutant is similar to wild type (Fig. 2.8), suggesting that if GA deficiency is one of the reasons for delayed flowering of *bhlh93* mutant, it might be localized in a tissue- or cell-specific manner, which contradicts the expression patterns of *bHLH93* as evidenced from the *pbHLH93:bHLH93-GUS* expression data (Fig. 2.12). Third, both semi-quantitative RT-PCR and whole genome expression profiling did not reveal any significant difference in gene expression for the GA biosynthetic and/or signaling genes between wt and *bhlh93* mutant (Fig. 2.9 Table 2.2). On the other hand, prolonged vernalization completely rescued the late flowering phenotype of *bhlh93* mutant (Fig. 2.10), suggesting that the vernalization pathway is not defective in this mutant. The lack of difference in expression of the majority of the vernalization pathway genes between wt and *bhlh93* mutant supports this hypothesis (Fig. 2.9; Table 2.2). By contrast, variations of different day-length showed that *bhlh93* mutants failed to flower as soon as the LD photoperiod pathway is turned off (e.g., the day-length is 12h or shorter) (Fig. 2.6). *bhlh93* mutants flowered, although later than wt controls, when the day-length is increased to 14h light, a condition where the LD photoperiod pathway is still operative.

Taken together, our results suggest that *bhlh93* is defective only under the non-inductive SD pathway as opposed to other genetic pathways regulating flowering time.

Although the genetic data provide compelling evidence that bHLH93 plays a pivotal role in regulating flowering time specifically under SD conditions, the cellular and molecular mechanisms by which it regulates flowering time remain to be elucidated. *bHLH93* is expressed at the meristematic tissues and primary leaves (Fig. 2.12 A-R), and the protein is localized to nucleus (Fig. 2.12S). Being a member of a well-characterized transcription factor family with transcriptional activation and homodimerization ability (Fig. 2.16), bHLH93 is expected to transcriptionally regulate gene expression. However, our semi-quantitative RT-PCR analyses of well-known candidate flowering genes as well as the whole genome expression profiling did not reveal any obvious difference in their mRNA expressions (Fig. 2.17; Fig. 2.9; Table 2.2). Interestingly, microarray data showed that a set of genes that has previously been implicated in regulating GA signaling and/or flowering time is either upregulated or downregulated in the *bhlh93* mutant relative to wild type (Fig. 2.17C). One possibility is that bHLH93 controls a number of genes moderately, and the *bhlh93* phenotype reflects the net result of upregulation of flowering time repressors and downregulation of flowering time inducers functioning in SD pathway. Alternatively, bHLH93 regulates gene expression at a later developmental stage or in a tissue- and/or cell type-specific manner that was not revealed in our whole genome expression profiling using 10-day old seedling aerial tissues. Because *bhlh93* mutants never flower under SD conditions, bHLH93 may constitute the SD pathway that regulates the facultative nature of flowering plants (Fig. 2.18). In this case, facultative plants may have evolved with regulators like bHLH93 that provide evolutionary advantage for reproductive success of facultative flowering behavior in varying

environmental conditions. Identification and characterization of direct targets of bHLH93 will help distinguish these possibilities.

ACCESSION NUMBERS

The microarray data have been deposited at Gene Expression Omnibus (GEO) under accession number GSE29786.

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Table 2.1: Primer sequences used in experiments described in the text

Gene	Forward	Reverse
For Genotyping		
bhlh93-1	TTTTCGATGGACGAATCTGTC	TACTGATTTTTGGGACGATGG
bhlh93-2	CAGAGGTTCGTTCGCATTAAG	TTAATGGCGGATTTGATCATC
T-DNA (*bHLH93*)	GCGTGGACCGCTTGCTGCAACT (LBb1)	CTCCTAATATCGATGTCCTGTC
For RT-PCR		
bHLH93	CAGGACATCGATATTAGGAGATGCC	CAAGCAGCTTCCACCATAACC
GID1a	CGAGCGATGAAGTTAATCT	GAAAACCCCATCAACCG
GID1b	GCTGGTGGTAACGAAG	ACGCAGGTTGGTAGAT
GAI	CAGATGCACTTCTACGAGACTTG	TCCCTTGAAAAGCTTCGAGAAT
RGA	CATTCCCGGAAACGCGATTTATCAG	TCACCGTCGTTCCTATGACTCCAC
FLC	TTCTCCAAACGTCGCAACGGTCTC	GATTTGTCCAGCAGGTGACATCTC
MAF5	AGTAATTGGGGATTAAGTCATAC	AACTCATCACCTTCCCTTTTTTTC
VIL1	ACACAGGTGGAGGTGCAGAAT	GGTTGGTCGCTGTCCACTTC
VIL2	AGTCCAACAACGATTACATTGTCCC	GTACAATGTCTGAGTCGGTTGTCC
SOC1	TGAGGCATACTAAGGATCGAGTCAG	GCGTCTACTTCAGAACTTGGGC
ELF7	TCTCAGATGCCCAAGGGACAC	GGATGCTTCAATATCCTTGATTTGT
EFS	CATCAAGTGAAAGTGCCGTGG	AGAGGATTTTCTCAGATGGCGAG
AGL15	GTCAAGCGATTCAGTGACAACAAAC	CAGAGAACCTTTGTCTTTTGGCTTC
AGL24	TCCATCGAAGTCAACTCTGCTGGATC	GTCTTCATGCAAGTAACATCAAC
AGL18	TAATCTCTTCTCTCTATATCTCTTCTC	AGATGAAATAAAGCAAAAGAACAGCCAG
AGL19	ATGGTGAGGGGCAAAACGGAG	CCAGATGTTTCGTCTCTCGC
SVP	ACTAACGGAAGAGAACGAGCGACTT	GGCGTTAGTAATAGACTCCGACGAC
CO	ACGCCATCAGCGAGTTCC	AAATGTATGCGTTATGGTTAATGG
FT	AGAAGACTTTAGATGGCTTCTT	TTATCGCATCACACACTATATAAG
UBQ10	GATCTTTGCCGGAAAACAATTGGAGGATGGT	CGACTTGTCATTAGAAAGAAAGAGATAACAGG
Cloning		
bHLH93:YFP tag	CACCATGGAACTGTCGACTCAAATG	CAAGCAGCTTCCACCATAACC
bHLH93:GUS tag	CCGGAAGCTTGTACTATATACACCCACACATG	CCTGGATCCCAAGCAGCTTCCACCATAACC
bHLH93:LUC tag	CCTGTCGACTTACAAGCAGCTTCCACCATAAC	AGAGAATTCATGGAACTGTCGACTCAAATG
Complementation	CCGGAAGCTTGTACTATATACACCCACACATG	CATTTGGATCTACATCAGTTGCCG

Table 2.2A: Genes up-regulated in dark

Gene	Gene Name	FC	q-value
AT3G32360	transposable element gene	3.4	0
AT5G33370	GDSL-motif lipase/hydrolase family protein	2.9	0
AT2G12540	transposable element gene	2.8	0
AT1G52680	late embryogenesis abundant protein-related / LEA protein-related	2.7	0
AT4G10112	unknown protein	2.7	0
AT3G29787	transposable element gene	2.7	0
AT4G39404	other RNA	2.7	0
AT1G78172	unknown protein	2.6	0
AT5G06080	LBD33 (LOB DOMAIN-CONTAINING PROTEIN 33)	2.6	0
AT5G23950	C2 domain-containing protein	2.5	0
AT4G10345	MIR832a; miRNA	2.4	0
AT2G27930	binding	2.4	0
AT3G46310	unknown protein	2.4	0
AT3G05193	unknown protein	2.4	0
AT4G09795	LCR13 (Low-molecular-weight cysteine-rich 13)	2.4	0
AT1G28307	unknown protein	2.3	0
AT5G46010	homeobox-leucine zipper transcription factor family protein	2.3	0
AT2G33670	MLO5 (MILDEW RESISTANCE LOCUS O 5)	2.2	0
AT5G52272	pseudogene of ACYB-2/ACYB-1 (cytochrome b reductase)	2.2	0
AT4G17710	HDG4 (HOMEODOMAIN GLABROUS 4)	2.2	0
AT3G03760	LBD20 (LOB DOMAIN-CONTAINING PROTEIN 20)	2.2	0
AT5G28865	transposable element gene	2.2	0
AT3G47180	zinc finger (C3HC4-type RING finger) family protein	2.2	0
AT1G66890	unknown protein	2.1	0
AT3G42916	transposable element gene	2.1	0
AT1G63060	unknown protein	2.0	0
AT2G41225	unknown protein	2.0	0
AT3G42466	Pseudogene of AT2G26730	2.0	0
AT3G05858	unknown protein	2.0	0
AT3G32035	transposable element gene	2.0	0
AT3G11773	electron carrier/ protein disulfide oxidoreductase	2.0	0
AT4G08333	transposable element gene	2.0	0
AT5G04310	pectate lyase family protein	2.0	0
AT4G24275	Identified as a screen for stress-responsive genes.	2.0	0

Table 2.2B: Genes down-regulated in dark

Gene	Gene Name	FC	q-value
AT1G23130	Bet v I allergen family protein	0.3	0
AT5G48120	binding	0.3	0
AT4G36210	unknown protein	0.3	0
AT3G05160	sugar transporter, putative	0.4	0
AT4G17880	basic helix-loop-helix (bHLH) family protein	0.4	0
AT5G10745	unknown protein	0.4	0
AT5G27290	unknown protein	0.4	0
AT4G17600	LIL3:1; transcription factor	0.4	0
AT2G27840	HDT4; histone deacetylase	0.4	0
AT3G45780	PHOT1 (PHOTOTROPIN 1)	0.4	0
AT4G31120	SKB1 (SHK1 BINDING PROTEIN 1)	0.4	0
AT1G08040	unknown protein	0.4	0
AT2G45830	DTA2 (DOWNSTREAM TARGET OF AGL15 2)	0.4	0
AT4G33580	carbonic anhydrase family protein	0.4	0
AT4G15020	DNA binding / protein dimerization	0.4	0
AT1G70770	unknown protein	0.4	0
AT4G38760	unknown protein	0.4	0
AT2G38695	unknown protein	0.4	0
AT3G61220	short-chain dehydrogenase/reductase (SDR) family protein	0.4	0
AT4G12560	F-box family protein	0.4	0
AT3G59320	integral membrane protein, putative	0.4	0
AT2G07727	cytochrome b (MTCYB) (COB) (CYTB)	0.4	0
AT5G35460	unknown protein	0.4	0
AT1G07470	transcription factor IIA large subunit	0.4	0
AT4G35470	leucine-rich repeat family protein	0.5	0
AT5G65960	unknown protein	0.5	0
AT4G27040	VPS22	0.5	0
AT3G22530	unknown protein	0.5	0
AT3G59300	unknown protein	0.5	0
AT3G07360	PUB9 (PLANT U-BOX 9)	0.5	0
AT5G50100	unknown protein	0.5	0
AT1G31812	ACBP6 (acyl-CoA-binding protein 6)	0.5	0
AT1G19920	APS2; sulfate adenylyltransferase (ATP)	0.5	0
AT3G22425	IGPD; imidazoleglycerol-phosphate dehydratase	0.5	0
AT4G24770	RBP31 (31-KDA RNA BINDING PROTEIN)	0.5	0
AT5G62390	ATBAG7 (ARABIDOPSIS THALIANA BCL-2-ASSOCIATED ATHANOGENE 7)	0.5	0
AT1G04520	PDLP2 (PLASMODESMATA-LOCATED PROTEIN 2)	0.5	0
AT5G57300	UbiE/COQ5 methyltransferase family protein	0.5	0
ATMG00400	ORF157	0.5	0

Table 2.2C: Genes up-regulated in light

Gene	Gene Name	FC	q-value
AT4G13830	J20 (DNAJ-LIKE 20); heat shock protein binding	3.3	0.00
AT2G25890	glycine-rich protein / oleosin	3.1	0.00
AT1G32045	transposable element gene	3.0	0.05
AT1G13670	unknown protein	3.0	0.05
AT3G50510	LBD28 (LOB DOMAIN-CONTAINING PROTEIN 28)	2.8	0.05
AT5G37710	lipase class 3 family protein	2.8	0.05
AT3G50651	unknown protein	2.7	0.05
AT3G04997	unknown protein	2.7	0.05
AT5G61180	unknown protein	2.7	0.00
AT1G30250	unknown protein	2.7	0.05
AT2G32280	unknown protein	2.7	0.05
AT5G24510	60s acidic ribosomal protein P1, putative	2.7	0.05
AT3G62400	unknown protein	2.6	0.05
AT5G11620	SWIM zinc finger family protein	2.6	0.05
AT3G26770	short-chain dehydrogenase/reductase (SDR) family protein	2.6	0.00
AT4G29276	Pseudogene of AT4G29270	2.6	0.05
AT3G14110	FLU (FLUORESCENT IN BLUE LIGHT); binding	2.6	0.05
AT1G35465	transposable element gene	2.6	0.05

Table 2.2C: Genes up-regulated in light (continued)

AT5G40310	exonuclease family protein	2.6	0.05
AT4G26780	AR192/adenyl-nucleotide exchange factor	2.5	0.00
AT1G60760	invertase/pectin methylesterase inhibitor family protein	2.5	0.00
AT1G08970	NF-YC9 (NUCLEAR FACTOR Y, SUBUNIT C9)	2.5	0.00
AT2G02135	Encodes a member of a family of small,secreted, cysteine rich protein with sequence similarity to the PCP (pollen coat protein) gene family.	2.5	0.05
AT2G24617	unknown protein	2.5	0.05
AT2G28140	unknown protein	2.5	0.05
AT5G37145	transposable element gene	2.5	0.05
AT5G37800	basic helix-loop-helix (bHLH) family protein	2.5	0.05
AT4G19920	disease resistance protein (TIR class), putative	2.5	0.05
AT4G40090	AGP3 (arabinogalactan-protein 3)	2.5	0.00
AT4G05095	unknown protein	2.4	0.05
AT1G19500	unknown protein	2.4	0.00
AT5G22870	harpin-induced protein-related	2.4	0.05
AT1G31690	amine oxidase/ copper ion binding / quinone binding	2.4	0.05
AT1G36770	transposable element gene	2.4	0.00
AT2G19610	zinc finger (C3HC4-type RING finger) family protein	2.4	0.05
AT5G56570	unknown protein	2.4	0.00
AT5G50830	unknown protein	2.4	0.05
AT2G03938	unknown protein	2.4	0.00
AT1G67350	unknown protein	2.4	0.05
AT1G43667	protease inhibitor/seed storage	2.3	0.05
AT4G12920	aspartyl protease family protein	2.3	0.05
AT5G27230	unknown protein	2.3	0.05
AT2G17723	Encodes a defensin-like (DEFL) family protein.	2.3	0.00
AT5G44570	unknown protein	2.3	0.00
AT2G14870	RNA recognition motif (RRM)-containing protein	2.3	0.00
AT4G12950	unknown protein	2.3	0.00
AT2G30010	unknown protein	2.3	0.05
AT4G08032	transposable element gene	2.3	0.05
AT1G52855	unknown protein	2.3	0.00
AT3G07510	unknown protein	2.3	0.00
AT1G73610	GDSL-motif lipase/hydrolase family protein	2.3	0.00
AT3G18120	F-box family protein-related	2.3	0.05
AT5G02320	MYB3R-5 (MYB DOMAIN PROTEIN 3R-5)	2.3	0.05
AT2G23620	MES1 (METHYL ESTERASE 1)	2.3	0.05
AT4G00190	pectinesterase family protein	2.3	0.05
AT2G19010	GDSL-motif lipase/hydrolase family protein	2.2	0.05
AT5G29070	Unknown protein	2.2	0.05
AT1G31245	Encodes a defensin-like (DEFL) family protein.	2.2	0.05

Table 2.2C: Genes up-regulated in light (continued)

AT1G56165	other RNA	2.1	0.05
AT5G24900	CYP714A2	2.0	0.05
AT5G22180	unknown protein	2.0	0.05
AT1G24140	matrixin family protein	2.0	0.05
AT3G45470	zinc finger protein-related	2.0	0.05
AT3G45570	zinc finger (C3HC4-type RING finger) family protein	2.0	0.05
ATMG00140	hypothetical protein	2.0	0.00
AT5G53850	haloacid dehalogenase-like hydrolase family protein	2.0	0.00
AT5G50480	NF-YC6 (NUCLEAR FACTOR Y, SUBUNIT C6)	2.0	0.05
AT5G27690	heavy-metal-associated domain-containing protein	2.0	0.00
AT2G10850	transposable element gene	2.0	0.05
AT5G27925	transposable element gene	2.0	0.05
AT5G04050	Unknown protein	2.0	0.00
AT5G08330	TCP family transcription factor, putative	2.0	0.05
AT3G48237	unknown protein	2.0	0.00
AT1G49010	myb family transcription factor	2.0	0.05
AT2G22145	Encodes a ECA1 gametogenesis related family protein	2.0	0.00
AT4G26370	antitermination NusB domain-containing protein	2.0	0.00
AT5G42203	unknown protein	2.0	0.05
AT5G46930	invertase/pectin methylesterase inhibitor family protein	2.0	0.05
AT3G19850	phototropic-responsive NPH3 family protein	2.0	0.05
AT3G24220	NCED6 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 6)	2.0	0.00
AT4G25260	invertase/pectin methylesterase inhibitor family protein	2.0	0.05
AT2G04290	transposable element gene	2.0	0.00

Table 2.2D: Genes down-regulated in light

Gene	Gene Name	FC	q-value
AT3G33002	RR2_LOTJA Chloroplast 30S ribosomal protein S2.	0.04	0
AT1G33860	unknown protein	0.05	0
AT4G06718	transposable element gene	0.08	0
AT4G06704	transposable element gene	0.08	0
AT3G42718	transposable element gene	0.09	0
AT2G13146	Pseudogene of AT2G12905	0.1	0
AT4G06738	transposable element gene	0.1	0
AT1G40104	unknown protein	0.1	0
AT5G35146	transposable element gene	0.1	0
AT5G32750	transposable element gene	0.1	0
AT3G42717	transposable element gene	0.1	0
AT2G07600	AJ312951 NADH dehydrogenase subunit F	0.1	0
AT2G11430	transposable element gene	0.1	0
AT2G14650	transposable element gene	0.1	0
AT2G01023	unknown protein	0.1	0
ATCG00930	tRNA-Ile	0.1	0
AT4G03840	transposable element gene	0.1	0
AT4G06607	transposable element gene	0.1	0
AT4G03650	transposable element gene	0.1	0
AT3G30802	transposable element gene	0.1	0
AT4G06730	transposable element gene	0.1	0
AT2G01022	transposable element gene	0.1	0
AT5G32071	transposable element gene	0.1	0
AT2G10690	transposable element gene	0.1	0
AT4G07519	transposable element gene	0.1	0
AT2G10175	transposable element gene	0.1	0
AT2G04670	transposable element gene	0.2	0
AT5G02815	pre-tRNA	0.2	0
AT1G52010	transposable element gene	0.2	0
AT4G08050	transposable element gene	0.2	0
ATCG00940	tRNA-Ala	0.2	0
AT1G38194	transposable element gene	0.2	0
ATCG00080	PSII I protein	0.2	0
ATCG00710	Encodes a 8 kD phosphoprotein that is a component of the photosystem II oxygen evolving core.	0.2	0
AT3G42253	transposable element gene	0.2	0
AT1G36305	transposable element gene	0.2	0

Table 2.2D: Genes down-regulated in light (continued)

AT5G32515	transposable element gene	0.2	0
AT3G29076	transposable element gene	0.2	0
AT3G29764	AJ012560 RNA polymerase	0.2	0
AT5G32125	transposable element gene	0.2	0
AT3G21990	receptor-like protein kinase-related	0.2	0
AT1G42605	transposable element gene	0.2	0
AT3G47280	transposable element gene	0.2	0
AT1G55625	pre-tRNA	0.2	0
AT2G07250	transposable element gene	0.2	0
AT3G33000	ATPI_TOBAC ATP synthase	0.2	0
AT1G79930	HSP91; ATP binding	0.2	0
AT4G07941	transposable element gene	0.2	0
AT3G33077	transposable element gene	0.2	0
AT1G36720	transposable element gene	0.2	0
AT4G07896	transposable element gene	0.2	0
AT3G31970	transposable element gene	0.2	0
AT2G12905	unknown protein	0.2	0
AT1G41720	transposable element gene	0.2	0
AT3G20362	unknown protein	0.2	0
AT5G32488	transposable element gene	0.2	0
AT2G24692	unknown protein	0.2	0
AT3G32000	transposable element gene	0.2	0
AT5G31981	transposable element gene	0.2	0
AT3G32880	transposable element gene	0.2	0
AT5G38705	transposable element gene	0.2	0
AT5G32475	transposable element gene	0.2	0
AT1G70050	pre-tRNA	0.2	0
AT1G25211	unknown protein	0.2	0
ATCG00130	ATPase F subunit.	0.2	0
ATCG00800	encodes a chloroplast ribosomal protein S3	0.2	0
AT2G09950	transposable element gene	0.2	0
AT5G32473	transposable element gene	0.2	0
AT4G07566	transposable element gene	0.2	0
ATCG00440	Encodes NADH dehydrogenase D3 subunit of the chloroplast NAD(P)H dehydrogenase complex	0.2	0
AT2G07640	D2,D4-dienoyl-CoA reductase-related	0.2	0
AT5G32423	transposable element gene	0.2	0
AT3G33085	transposable element gene	0.2	0
AT3G32975	transposable element gene	0.2	0
AT1G76330	pre-tRNA	0.2	0
AT2G12680	transposable element gene	0.2	0

Table 2.2D: Genes down-regulated in light (continued)

ATCG00040	Encodes a maturase located in the trnK intron in the chloroplast genome.	0.2	0
AT2G02190	transposable element gene	0.2	0
AT3G32970	transposable element gene	0.2	0
AT5G32107	transposable element gene	0.2	0
AT4G06506	transposable element gene	0.2	0
AT3G37820	transposable element gene	0.2	0
AT4G08695	AF123794 ribosomal protein L2	0.2	0
AT4G07456	transposable element gene	0.2	0
AT3G33124	transposable element gene	0.2	0
AT4G06573	transposable element gene	0.2	0
AT5G32511	transposable element gene	0.2	0
AT2G06250	transposable element gene	0.2	0
AT4G06666	transposable element gene	0.2	0
AT5G33254	transposable element gene	0.2	0
AT2G13010	transposable element gene	0.2	0
AT4G03826	transposable element gene	0.2	0
AT3G42730	transposable element gene	0.2	0
AT5G33050	transposable element gene	0.2	0
AT2G09187	transposable element gene	0.2	0
AT1G55400	transposable element gene	0.2	0
AT5G35021	transposable element gene	0.2	0
AT2G15190	transposable element gene	0.2	0
AT3G33080	transposable element gene	0.2	0
AT4G10580	transposable element gene	0.2	0
AT3G51640	unknown protein	0.2	0
AT2G14180	transposable element gene	0.2	0
AT1G38450	transposable element gene	0.2	0
AT1G42050	transposable element gene	0.2	0
AT1G36520	transposable element gene	0.2	0
AT1G21110	O-methyltransferase, putative	0.2	0
AT4G06720	transposable element gene	0.2	0
AT3G32397	transposable element gene	0.2	0
AT2G16820	transposable element gene	0.2	0
AT1G40141	transposable element gene	0.2	0
AT3G33067	transposable element gene	0.2	0
AT1G58380	XW6; structural constituent of ribosome	0.2	0
ATCG01080	NADH dehydrogenase ND6	0.2	0
AT4G03850	transposable element gene	0.2	0
AT5G29571	transposable element gene	0.2	0
ATCG00520	Encodes a protein required for photosystem I assembly and stability.	0.2	0

Table 2.2D: Genes down-regulated in light (continued)

AT2G09930	transposable element gene	0.2	0
AT3G01500	CA1 (CARBONIC ANHYDRASE 1)	0.2	0
AT5G33000	transposable element gene	0.2	0
AT3G32220	transposable element gene	0.2	0
AT3G31630	transposable element gene	0.3	0
ATCG00300	encodes PsbZ, which is a subunit of photosystem II	0.3	0
AT2G12260	transposable element gene	0.3	0
AT5G31855	transposable element gene	0.3	0
AT1G40077	transposable element gene	0.3	0
AT2G08986	unknown protein	0.3	0
AT2G18323	Pseudogene of ATMG00450	0.3	0
AT1G41680	transposable element gene	0.3	0
AT3G10100	transposable element gene	0.3	0
AT5G31770	transposable element gene	0.3	0
AT5G56605	transposable element gene	0.3	0
AT4G06579	transposable element gene	0.3	0
AT4G08114	transposable element gene	0.3	0
AT3G32060	transposable element gene	0.3	0
AT5G32512	transposable element gene	0.3	0
AT4G06605	transposable element gene	0.3	0
AT2G42230	tubulin-specific chaperone C-related	0.3	0
AT5G32197	transposable element gene	0.3	0
AT1G34700	transposable element gene	0.3	0
AT2G10720	transposable element gene	0.3	0
AT5G34990	transposable element gene	0.3	0
AT4G08102	transposable element gene	0.3	0
AT5G33434	transposable element gene	0.3	0
AT1G42370	transposable element gene	0.3	0
AT2G12740	transposable element gene	0.3	0
AT3G48070	protein binding / zinc ion binding	0.3	0
AT3G42236	transposable element gene	0.3	0
AT3G30396	transposable element gene	0.3	0
AT2G07732	ribulose-bisphosphate carboxylase	0.3	0
AT4G06726	transposable element gene	0.3	0
AT2G14780	transposable element gene	0.3	0
AT5G61290	flavin-containing monooxygenase family protein	0.3	0
AT2G14230	transposable element gene	0.3	0
AT4G08991	Pseudogene of AT4G14145	0.3	0
AT1G03850	glutaredoxin family protein	0.3	0
AT2G11500	transposable element gene	0.3	0
AT5G36840	transposable element gene	0.3	0

Table 2.2D: Genes down-regulated in light (continued)

AT3G43862	transposable element gene	0.3	0
AT1G29930	CAB1 (CHLOROPHYLL A/B BINDING PROTEIN 1)	0.3	0
AT3G32415	transposable element gene	0.3	0
AT1G39750	transposable element gene	0.3	0
AT1G13245	RTFL17 (ROTUNDIFOLIA LIKE 17)	0.3	0
AT5G31572	transposable element gene	0.3	0
AT2G10490	transposable element gene	0.3	0
AT1G42375	transposable element gene	0.3	0
AT4G06561	transposable element gene	0.3	0
AT4G06517	transposable element gene	0.3	0
AT5G34890	transposable element gene	0.3	0
AT2G06460	transposable element gene	0.3	0
ATCG00460	Chloroplast encoded tRNA-Met gene.	0.3	0
AT3G33058	transposable element gene	0.3	0
AT4G06485	transposable element gene	0.3	0
AT3G32210	transposable element gene	0.3	0
ATMG00650	Encodes NADH dehydrogenase subunit 4L.	0.3	0
AT5G32505	transposable element gene	0.3	0
AT1G38460	transposable element gene	0.3	0
AT4G08115	transposable element gene	0.3	0
AT1G41650	transposable element gene	0.3	0
AT1G49340	1-phosphatidylinositol 4-kinase	0.3	0
AT3G43864	transposable element gene	0.3	0
AT3G32391	transposable element gene	0.3	0
AT3G32899	transposable element gene	0.3	0
AT3G30827	transposable element gene	0.3	0
AT3G31330	transposable element gene	0.3	0
ATCG00900	encodes a chloroplast ribosomal protein S7, a constituent of the small subunit of the ribosomal complex	0.3	0
AT2G07260	transposable element gene	0.3	0
AT4G22785	pre-tRNA	0.3	0
AT3G24612	snoRNA	0.3	0
AT3G32020	transposable element gene	0.3	0
AT1G37537	transposable element gene	0.3	0
AT4G06728	transposable element gene	0.3	0
AT2G06330	transposable element gene	0.3	0
AT5G46250	RNA recognition motif (RRM)-containing protein	0.3	0
AT1G25784	transposable element gene	0.3	0
AT1G40150	transposable element gene	0.3	0
AT3G31357	transposable element gene	0.3	0
AT4G05306	transposable element gene	0.3	0

Table 2.2D: Genes down-regulated in light (continued)

AT1G23130	Bet v I allergen family protein	0.3	0
AT5G56010	HSP81-3; ATP binding / unfolded protein binding	0.3	0
AT3G60950	Unknown protein	0.3	0
AT1G36120	transposable element gene	0.3	0
AT1G43240	transposable element gene	0.3	0
AT5G40910	disease resistance protein (TIR-NBS-LRR class), putative	0.3	0
AT1G40117	transposable element gene	0.3	0
AT1G75560	zinc knuckle (CCHC-type) family protein	0.3	0
AT2G39780	RNS2 (RIBONUCLEASE 2)	0.3	0
AT3G42763	transposable element gene	0.3	0
AT2G14790	transposable element gene	0.3	0
AT2G02210	transposable element gene	0.3	0
AT4G07355	transposable element gene	0.3	0
AT4G06505	transposable element gene	0.3	0
AT3G30695	transposable element gene	0.3	0
AT2G07827	unknown protein	0.3	0
AT2G14130	transposable element gene	0.3	0
AT2G11115	transposable element gene	0.3	0
AT3G30700	transposable element gene	0.3	0
AT3G30668	transposable element gene	0.3	0
AT5G32516	transposable element gene	0.3	0
AT1G42365	transposable element gene	0.3	0
AT4G28250	ATEXPB3 (ARABIDOPSIS THALIANA EXPANSIN B3)	0.3	0
AT1G41700	transposable element gene	0.3	0
AT2G07660	transposable element gene	0.3	0
AT5G57650	eukaryotic translation initiation factor-related	0.3	0
AT4G07640	transposable element gene	0.3	0
AT1G38149	transposable element gene	0.3	0
AT1G27815	transposable element gene	0.3	0
AT3G18240	unknown protein	0.3	0
AT4G06562	transposable element gene	0.3	0
AT3G01500	CA1 (CARBONIC ANHYDRASE 1)	0.3	0
AT4G08880	transposable element gene	0.3	0
AT4G07830	transposable element gene	0.3	0
ATCG00680	encodes for CP47, subunit of the photosystem II reaction center.	0.3	0
AT1G40070	transposable element gene	0.3	0
AT3G47310	transposable element gene	0.3	0
AT1G23310	GGT1 (GLUTAMATE:GLYOXYLATE AMINOTRANSFERASE)	0.3	0
AT4G06575	transposable element gene	0.3	0

Table 2.2D: Genes down-regulated in light (continued)

AT5G01015	unknown protein	0.3	0
AT5G35046	transposable element gene	0.3	0
AT3G31960	Pseudogene of AT3G31960	0.3	0
AT2G30850	pre-tRNA	0.3	0
AT1G29620	unknown protein	0.3	0
AT2G06303	transposable element gene	0.3	0
AT4G36360	BGAL3 (beta-galactosidase 3)	0.3	0
AT2G09990	40S ribosomal protein S16 (RPS16A)	0.3	0
AT4G03405	pre-tRNA	0.3	0
AT1G06680	PSBP-1 (PHOTOSYSTEM II SUBUNIT P-1)	0.3	0
AT1G37060	transposable element gene	0.3	0
AT5G07340	calnexin, putative	0.3	0
AT1G39910	transposable element gene	0.3	0
AT1G10130	ECA3 (ENDOPLASMIC RETICULUM-TYPE CALCIUM-TRANSPORTING ATPASE 3)	0.3	0
AT1G24825	other RNA	0.3	0
AT2G06600	transposable element gene	0.3	0
AT2G06370	transposable element gene	0.3	0
AT5G28263	transposable element gene	0.3	0
AT3G19000	oxidoreductase	0.3	0
AT4G06722	transposable element gene	0.3	0
AT1G41770	transposable element gene	0.3	0
AT2G22280	pre-tRNA	0.3	0
AT1G36460	transposable element gene	0.3	0
AT1G37160	transposable element gene	0.3	0
AT2G38260	AF165158 carboxyl transferase alpha subunit	0.3	0
AT1G39270	transposable element gene	0.4	0
AT1G37063	transposable element gene	0.4	0
AT2G07664	Pseudogene of ATMG01040	0.4	0
AT1G19392	transposable element gene	0.4	0
AT5G61050	histone deacetylase-related / HD-related	0.4	0
AT4G08138	transposable element gene	0.4	0
AT1G65270	unknown protein	0.4	0
AT2G06310	transposable element gene	0.4	0
AT1G54730	sugar transporter, putative	0.4	0
AT1G38410	transposable element gene	0.4	0
AT2G05570	transposable element gene	0.4	0
AT3G14390	diaminopimelate decarboxylase, putative	0.4	0
AT2G05140	phosphoribosylaminoimidazole carboxylase family protein	0.4	0
AT5G38410	ribulose bisphosphate carboxylase small chain 3B	0.4	0
AT1G24851	unknown protein	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT1G21160	GTP binding / GTPase/ translation initiation factor	0.4	0
AT5G24360	IRE1-1 (INOSITOL REQUIRING 1-1)	0.4	0
AT1G12935	transposable element gene	0.4	0
AT5G62700	TUB3; GTP binding / GTPase/ structural molecule	0.4	0
AT2G06470	transposable element gene	0.4	0
AT2G20810	GAUT10 (GALACTURONOSYLTRANSFERASE 10)	0.4	0
AT3G29480	transposable element gene	0.4	0
ATCG01230	chloroplast gene encoding ribosomal protein s12.	0.4	0
AT2G45960	PIP1B (NAMED PLASMA MEMBRANE INTRINSIC PROTEIN 1B)	0.4	0
AT5G63400	ADK1 (ADENYLATE KINASE 1)	0.4	0
AT3G32677	transposable element gene	0.4	0
AT1G53500	MUM4 (MUCILAGE-MODIFIED 4)	0.4	0
AT3G44310	NIT1; indole-3-acetonitrile nitrilase	0.4	0
AT1G55710	unknown protein	0.4	0
AT1G40119	transposable element gene	0.4	0
AT1G36470	transposable element gene	0.4	0
AT2G10280	transposable element gene	0.4	0
AT4G06622	transposable element gene	0.4	0
AT2G06245	transposable element gene	0.4	0
AT3G05030	NHX2 (SODIUM HYDROGEN EXCHANGER 2)	0.4	0
AT1G43444	transposable element gene	0.4	0
AT2G25610	H+-transporting two-sector ATPase, C subunit family protein	0.4	0
AT1G41690	transposable element gene	0.4	0
AT1G55546	unknown protein	0.4	0
AT5G28923	transposable element gene	0.4	0
ATCG00580	PSII cytochrome b559	0.4	0
AT4G06724	transposable element gene	0.4	0
AT5G56100	glycine-rich protein / oleosin	0.4	0
AT5G63370	protein kinase family protein	0.4	0
AT1G36850	transposable element gene	0.4	0
AT5G08030	glycerophosphoryl diester phosphodiesterase family protein	0.4	0
AT2G01024	transposable element gene	0.4	0
AT2G01034	transposable element gene	0.4	0
AT4G06543	transposable element gene	0.4	0
AT5G38410	ribulose bisphosphate carboxylase small chain 3B	0.4	0
ATCG00480	chloroplast-encoded gene for beta subunit of ATP synthase	0.4	0
AT2G07703	transposable element gene	0.4	0
AT5G31909	pseudogene, hypothetical protein	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT4G07458	transposable element gene	0.4	0
AT4G02490	transposable element gene	0.4	0
AT4G37500	xanthine dehydrogenase family protein	0.4	0
AT5G64940	ATATH13; transporter	0.4	0
AT5G33624	transposable element gene	0.4	0
AT2G05930	transposable element gene	0.4	0
AT3G01500	CA1 (CARBONIC ANHYDRASE 1)	0.4	0
AT5G18110	NCBP (NOVEL CAP-BINDING PROTEIN)	0.4	0
AT4G24740	AFC2 (ARABIDOPSIS FUS3-COMPLEMENTING GENE 2)	0.4	0
AT4G34050	caffeoyl-CoA 3-O-methyltransferase, putative	0.4	0
AT4G11830	PLDGAMMA2; phospholipase D	0.4	0
AT1G40101	transposable element gene	0.4	0
AT2G18960	AHA1 (ARABIDOPSIS H+ ATPASE 1)	0.4	0
AT4G03790	transposable element gene	0.4	0
AT2G09900	transposable element gene	0.4	0
AT3G53342	unknown protein	0.4	0
AT5G49720	ATGH9A1 (ARABIDOPSIS THALIANA GLYCOSYL HYDROLASE 9A1)	0.4	0
AT1G32820	transposable element gene	0.4	0
AT1G74456	snoRNA	0.4	0
AT5G32486	transposable element gene	0.4	0
AT4G02820	pentatricopeptide (PPR) repeat-containing protein	0.4	0
ATCG00350	Encodes psaA protein comprising the reaction center for photosystem I along with psaB protein; hydrophobic protein encoded by the chloroplast genome.	0.4	0
AT4G11850	PLDGAMMA1; phospholipase D	0.4	0
AT2G42240	nucleic acid binding / nucleotide binding	0.4	0
AT4G08660	transposable element gene	0.4	0
AT1G02840	SR1; RNA binding / nucleic acid binding / nucleotide binding	0.4	0
AT2G12840	transposable element gene	0.4	0
AT2G10480	transposable element gene	0.4	0
ATCG00905	chloroplast gene encoding ribosomal protein s12	0.4	0
AT3G42996	transposable element gene	0.4	0
AT1G40072	transposable element gene	0.4	0
AT5G32402	transposable element gene	0.4	0
AT3G22121	other RNA	0.4	0
AT2G13830	transposable element gene	0.4	0
AT2G11910	unknown protein	0.4	0
AT1G18400	BEE1 (BR Enhanced Expression 1)	0.4	0
AT3G13460	ECT2; protein binding	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT5G27190	transposable element gene	0.4	0
AT4G31680	transcriptional factor B3 family protein	0.4	0
AT3G33073	transposable element gene	0.4	0
AT2G07811	pseudogene of mitochondrial protein	0.4	0
AT1G11860	aminomethyltransferase, putative	0.4	0
AT1G37015	transposable element gene	0.4	0
AT1G43995	transposable element gene	0.4	0
AT1G03570	pre-tRNA	0.4	0
AT5G26236	transposable element gene	0.4	0
AT5G50380	ATEXO70F1 (exocyst subunit EXO70 family protein F1)	0.4	0
AT5G29075	transposable element gene	0.4	0
AT3G43152	transposable element gene	0.4	0
AT1G31580	ECS1	0.4	0
AT5G26670	pectinacetylesterase, putative	0.4	0
AT1G14940	major latex protein-related / MLP-related	0.4	0
AT4G04393	transposable element gene	0.4	0
AT2G27840	HDT4; histone deacetylase	0.4	0
AT2G33340	nucleotide binding / ubiquitin-protein ligase	0.4	0
AT2G38810	HTA8 (HISTONE H2A 8); DNA binding	0.4	0
AT1G19400	unknown protein	0.4	0
AT1G29910	CAB3 (CHLOROPHYLL A/B BINDING PROTEIN 3)	0.4	0
AT3G30418	transposable element gene	0.4	0
AT5G02500	HSC70-1 (HEAT SHOCK COGNATE PROTEIN 70-1)	0.4	0
AT2G14980	transposable element gene	0.4	0
AT3G42621	transposable element gene	0.4	0
AT5G29295	transposable element gene	0.4	0
AT3G09200	60S acidic ribosomal protein P0 (RPP0B)	0.4	0
AT2G12110	transposable element gene	0.4	0
AT5G22030	UBP8 (UBIQUITIN-SPECIFIC PROTEASE 8)	0.4	0
AT1G03935	snoRNA	0.4	0
AT2G14770	transposable element gene	0.4	0
AT1G13650	Unknown protein	0.4	0
AT1G76930	ATEXT4 (EXTENSIN 4)	0.4	0
AT4G06624	transposable element gene	0.4	0
AT5G29408	transposable element gene	0.4	0
AT5G60250	zinc finger (C3HC4-type RING finger) family protein	0.4	0
AT3G30665	transposable element gene	0.4	0
AT4G01037	ubiquitin thiolesterase	0.4	0
AT1G40118	transposable element gene	0.4	0
AT3G42254	transposable element gene	0.4	0
AT5G28540	BIP1; ATP binding	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT1G07930	elongation factor 1-alpha / EF-1-alpha	0.4	0
ATCG00140	ATPase III subunit	0.4	0
AT4G17880	basic helix-loop-helix (bHLH) family protein	0.4	0
AT1G36795	transposable element gene	0.4	0
AT5G05170	CEV1 (CONSTITUTIVE EXPRESSION OF VSP 1)	0.4	0
AT3G32393	transposable element gene	0.4	0
AT1G41890	AB022783 amino acid permease	0.4	0
AT4G10480	nascent polypeptide associated complex alpha chain protein	0.4	0
AT3G29695	transposable element gene	0.4	0
AT1G07780	PAI1 (PHOSPHORIBOSYLANTHRANILATE ISOMERASE 1)	0.4	0
AT3G14420	(S)-2-hydroxy-acid oxidase, peroxisomal, putative	0.4	0
AT5G59710	VIP2 (VIRE2 INTERACTING PROTEIN2)	0.4	0
AT1G38423	transposable element gene	0.4	0
AT1G69480	EXS family protein / ERD1/XPR1/SYG1 family protein	0.4	0
AT3G10980	unknown protein	0.4	0
AT2G13390	transposable element gene	0.4	0
AT1G49630	ATPREP2 (ARABIDOPSIS THALIANA PRESEQUENCE PROTEASE 2)	0.4	0
AT3G54470	uridine 5'-monophosphate synthase / UMP synthase (PYRE-F)	0.4	0
ATCG00280	chloroplast gene encoding a CP43 subunit of the photosystem II reaction center. promoter contains a blue-light responsive element.	0.4	0
AT3G44310	NIT1; indole-3-acetonitrile nitrilase	0.4	0
AT4G08131	transposable element gene	0.4	0
AT2G46280	TRIP-1 (TGF-BETA RECEPTOR INTERACTING PROTEIN 1)	0.4	0
AT2G44670	senescence-associated protein-related	0.4	0
AT4G06580	transposable element gene	0.4	0
AT1G70505	unknown protein	0.4	0
AT3G44400	disease resistance protein (TIR-NBS-LRR class), putative	0.4	0
AT5G18570	GTP1/OBG family protein	0.4	0
ATCG00500	Encodes the carboxytransferase beta subunit of the Acetyl-CoA carboxylase (ACCase) complex in plastids.	0.4	0
AT5G29041	transposable element gene	0.4	0
AT2G38010	ceramidase family protein	0.4	0
AT4G40060	ATHB16 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 16)	0.4	0
ATMG00500	hypothetical protein	0.4	0
AT5G66562	snoRNA	0.4	0
AT5G04800	40S ribosomal protein S17 (RPS17D)	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT2G41600	Unknown protein	0.4	0
AT2G35680	dual specificity protein phosphatase family protein	0.4	0
AT1G25054	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	0.4	0
AT4G02715	unknown protein	0.4	0
AT1G14800	Unknown protein	0.4	0
AT4G24620	PGI1 (PHOSPHOGLUCOSE ISOMERASE 1); glucose-6-phosphate isomerase	0.4	0
AT5G60360	AALP (Arabidopsis aleurain-like protease)	0.4	0
AT5G27770	60S ribosomal protein L22 (RPL22C)	0.4	0
AT5G07250	ATRBL3 (ARABIDOPSIS RHOMBOID-LIKE PROTEIN 3)	0.4	0
AT5G63840	RSW3 (RADIAL SWELLING 3)	0.4	0
AT5G45775	60S ribosomal protein L11 (RPL11D)	0.4	0
AT2G14320	transposable element gene	0.4	0
AT1G14670	endomembrane protein 70, putative	0.4	0
AT1G52960	transposable element gene	0.4	0
AT1G19400	unknown protein	0.4	0
AT1G40105	transposable element gene	0.4	0
AT4G16950	RPP5 (RECOGNITION OF PERONOSPORA PARASITICA 5)	0.4	0
AT2G45810	DEAD/DEAH box helicase, putative	0.4	0
AT5G58320	kinase interacting protein-related	0.4	0
AT5G15230	GASA4 (GAST1 PROTEIN HOMOLOG 4)	0.4	0
AT5G63570	GSA1 (GLUTAMATE-1-SEMIALDEHYDE-2,1-AMINOMUTASE)	0.4	0
AT3G30690	transposable element gene	0.4	0
AT4G06752	transposable element gene	0.4	0
AT3G46487	transposable element gene	0.4	0
AT3G46320	histone H4	0.4	0
AT3G33091	transposable element gene	0.4	0
AT1G41730	transposable element gene	0.4	0
AT2G22090	UBP1 interacting protein 1a (UBA1a)	0.4	0
AT2G04280	unknown protein	0.4	0
AT4G14716	ATARD1 (ACIREDUCTONE DIOXYGENASE 1)	0.4	0
AT5G13000	ATGSL12 (glucan synthase-like 12)	0.4	0
AT2G34400	pentatricopeptide (PPR) repeat-containing protein	0.4	0
AT1G70310	SPDS2 (spermidine synthase 2)	0.4	0
AT3G48560	CSR1 (CHLORSULFURON/IMIDAZOLINONE RESISTANT 1)	0.4	0
AT2G37710	RLK (receptor lectin kinase)	0.4	0
AT5G32143	transposable element gene	0.4	0

Table 2.2D: Genes down-regulated in light (continued)

AT5G51200	unknown protein	0.4	0
AT3G54590	ATHRGP1 (HYDROXYPROLINE-RICH GLYCOPROTEIN)	0.4	0
AT2G10770	transposable element gene	0.4	0
AT2G12810	transposable element gene	0.4	0
AT5G01500	mitochondrial substrate carrier family protein	0.4	0
AT3G42313	transposable element gene	0.4	0
AT2G11050	transposable element gene	0.4	0
AT5G67560	ATARLA1D (ADP-ribosylation factor-like A1D)	0.4	0
AT3G32394	transposable element gene	0.4	0
AT3G31490	transposable element gene	0.4	0
AT2G24910	transposable element gene	0.5	0
AT3G30505	transposable element gene	0.5	0
AT1G80940	unknown protein	0.5	0
AT5G18230	transcription regulator NOT2/NOT3/NOT5 family protein	0.5	0
AT4G21705	pentatricopeptide (PPR) repeat-containing protein	0.5	0
AT3G22120	CWLP (CELL WALL-PLASMA MEMBRANE LINKER PROTEIN)	0.5	0
AT4G07492	transposable element gene	0.5	0
AT1G62630	disease resistance protein (CC-NBS-LRR class), putative	0.5	0
AT1G52730	transducin family protein / WD-40 repeat family protein	0.5	0
AT5G64320	pentatricopeptide (PPR) repeat-containing protein	0.5	0
AT1G12830	unknown protein	0.5	0
AT1G08640	unknown protein	0.5	0
AT3G47350	short-chain dehydrogenase/reductase (SDR) family protein	0.5	0
AT1G61440	S-locus protein kinase, putative	0.5	0
AT1G51680	4CL1 (4-COUMARATE:COA LIGASE 1)	0.5	0
AT1G36763	transposable element gene	0.5	0
AT5G04140	GLU1 (GLUTAMATE SYNTHASE 1)	0.5	0
AT4G25386	unknown protein	0.5	0
AT4G19290	transposable element gene	0.5	0
AT2G45180	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0.5	0
ATCG00670	Encodes the only ClpP (caseinolytic protease) encoded within the plastid genome.	0.5	0
AT3G13920	EIF4A1 (EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1)	0.5	0
AT5G36658	Encodes a ECA1 gametogenesis related family protein	0.5	0
AT1G37275	transposable element gene	0.5	0
AT4G03900	transposable element gene	0.5	0
AT3G08720	S6K2 (ARABIDOPSIS THALIANA SERINE/THREONINE PROTEIN KINASE 2)	0.5	0

Table 2.2D: Genes down-regulated in light (continued)

AT5G38660	APE1 (ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT)	0.5	0
AT1G66180	aspartyl protease family protein	0.5	0
AT3G60990	unknown protein	0.5	0
AT1G40071	transposable element gene	0.5	0
AT1G15690	AVP1; ATPase/ hydrogen-translocating pyrophosphatase	0.5	0
AT2G06630	transposable element gene	0.5	0
AT3G04400	emb2171 (embryo defective 2171)	0.5	0
AT4G06483	transposable element gene	0.5	0
AT5G60990	DEAD/DEAH box helicase, putative (RH10)	0.5	0
AT3G16640	TCTP (TRANSLATIONALLY CONTROLLED TUMOR PROTEIN)	0.5	0
AT1G03506	snoRNA	0.5	0
AT1G45211	Encodes a ECA1 gametogenesis related family protein [pseudogene]	0.5	0
AT5G11580	UVB-resistance protein-related	0.5	0
AT5G23630	ATPase E1-E2 type family protein	0.5	0
AT1G37130	NIA2 (NITRATE REDUCTASE 2)	0.5	0
AT4G32450	pentatricopeptide (PPR) repeat-containing protein	0.5	0
AT3G62750	BGLU8 (BETA GLUCOSIDASE 8)	0.5	0
AT4G03780	transposable element gene	0.5	0
AT1G09900	pentatricopeptide (PPR) repeat-containing protein	0.5	0
AT5G49760	leucine-rich repeat family protein / protein kinase family protein	0.5	0
AT2G18610	unknown protein	0.5	0
AT1G11860	aminomethyltransferase, putative	0.5	0
AT2G06430	transposable element gene	0.5	0
AT1G66990	AL606993 OSJNBb0051N19.7	0.5	0
AT2G10310	transposable element gene	0.5	0
AT5G12370	SEC10 (EXOCYST COMPLEX COMPONENT SEC10)	0.5	0
AT4G12790	ATP-binding family protein	0.5	0
AT5G61770	PPAN (PETER PAN-LIKE PROTEIN)	0.5	0
AT1G43750	transposable element gene	0.5	0
AT4G03981	transposable element gene	0.5	0
AT5G50580	SAE1B (SUMO-ACTIVATING ENZYME 1B)	0.5	0
AT1G36210	transposable element gene	0.5	0
AT1G42320	transposable element gene	0.5	0
AT1G12900	GAPA-2 (GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT 2)	0.5	0
AT2G28670	disease resistance-responsive family protein / fibroin-related	0.5	0
AT3G15602	transposable element gene	0.5	0

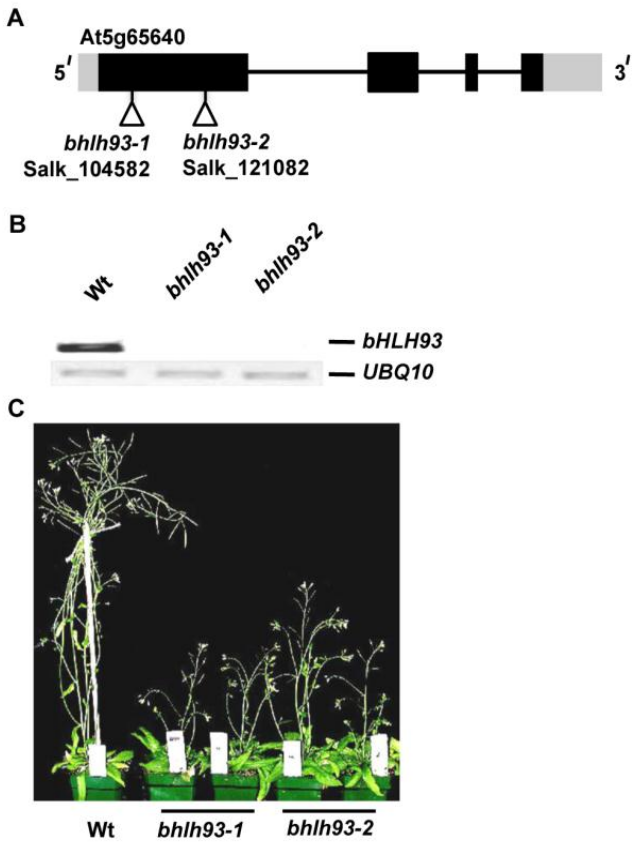

Figure 2.1: Adult phenotypes of *bhlh93* T-DNA insertion mutants.

A) Schematic diagram of the *bHLH93* gene structure showing four exons (black rectangles) connected with three introns (black lines) and 5'- and 3'-untranslated regions (gray rectangles). Two T-DNA alleles (*bhlh93-1* and *bhlh93-2*) have T-DNA insertion in the first exon. B) Semi-quantitative RT-PCR shows both *bhlh93-1* and *bhlh93-2* alleles are null mutants where *bHLH93* is not expressed. C) Adult morphological phenotypes of *bhlh93* mutant alleles along with wt controls grown under continuous light in a growth room. *bhlh93* mutant shows stunted growth with twisted leaves as compared to wild type. n>15

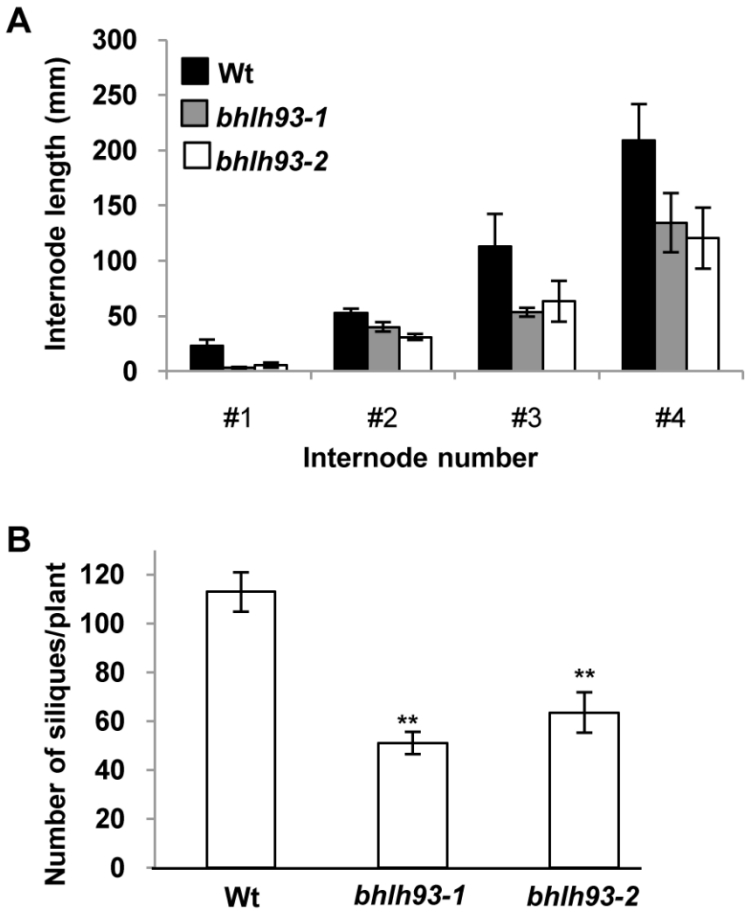

Figure 2.2: Adult phenotypes of the *bhlh93* mutants compared to wt plants.

Internode length (A) and number of siliques/plant (B) is shown for wild type and two alleles of *bhlh93* mutant grown under continuous light in green house conditions. **, indicates statistically significant difference with a p value $\leq$0.01. n>15

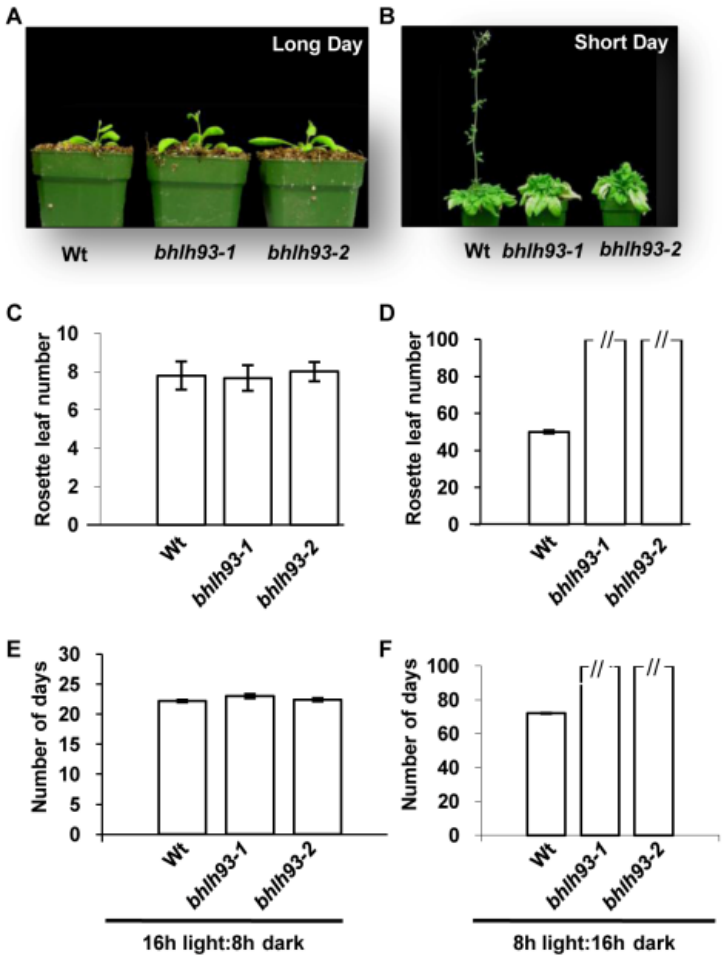

Figure 2.3: *bhlh93* does not flower specifically under short day (SD) conditions.

Flowering time was quantified using both number of rosette leaves formed at the time of flowering and number of days to flower. Photographs of wt and two alleles of *bhlh93* mutant plants grown under LD (A) or SD (B) conditions. Rosette leaf numbers for wt and two alleles of *bhlh93* mutant plants grown under LD (C) or SD (D) conditions. Number of days taken to flower for wt and two alleles of *bhlh93* mutant plants grown under LD (E) or SD (F) conditions. n>15

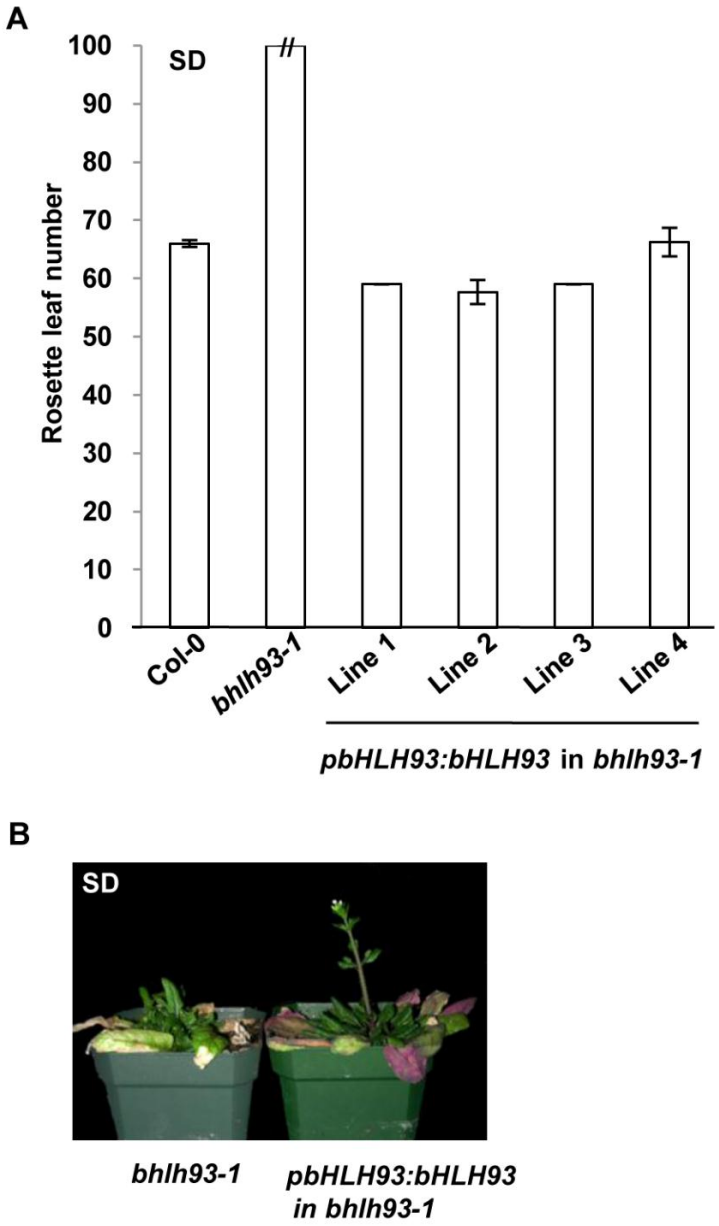

Figure 2.4: Complementation of *bhlh93* mutant with *pbHLH93:bHLH93*.

A) Bar graph shows the number of rosette leaves formed at the time of bolting for wt, *bhlh93* and four independent complementation lines grown under SD conditions. B) Photograph of *bhlh93* mutant and one complemented line showing emergence of an inflorescence from the primary meristem. n>15

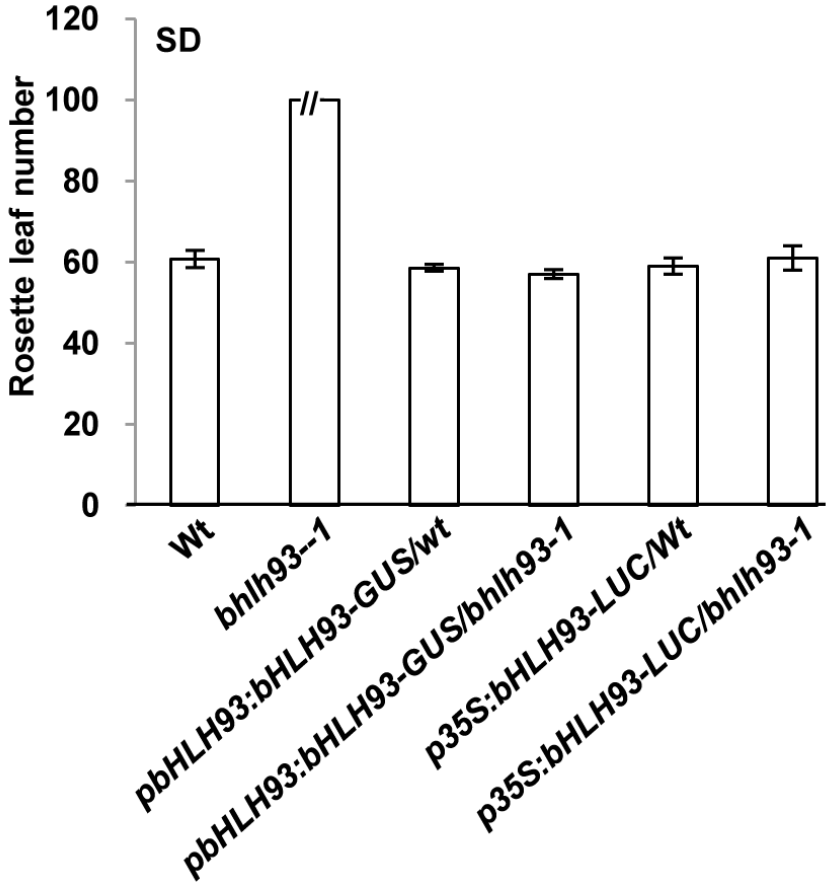

Figure 2.5: Overexpression of *pbHLH93:bHLH93-GUS* and *p35S:bHLH93-LUC* in wt background.

Overexpression of bHLH93 in transgenic lines did not accelerate flowering time under SD conditions. Bar graph shows the number of rosette leaves formed at the time of bolting. n>15

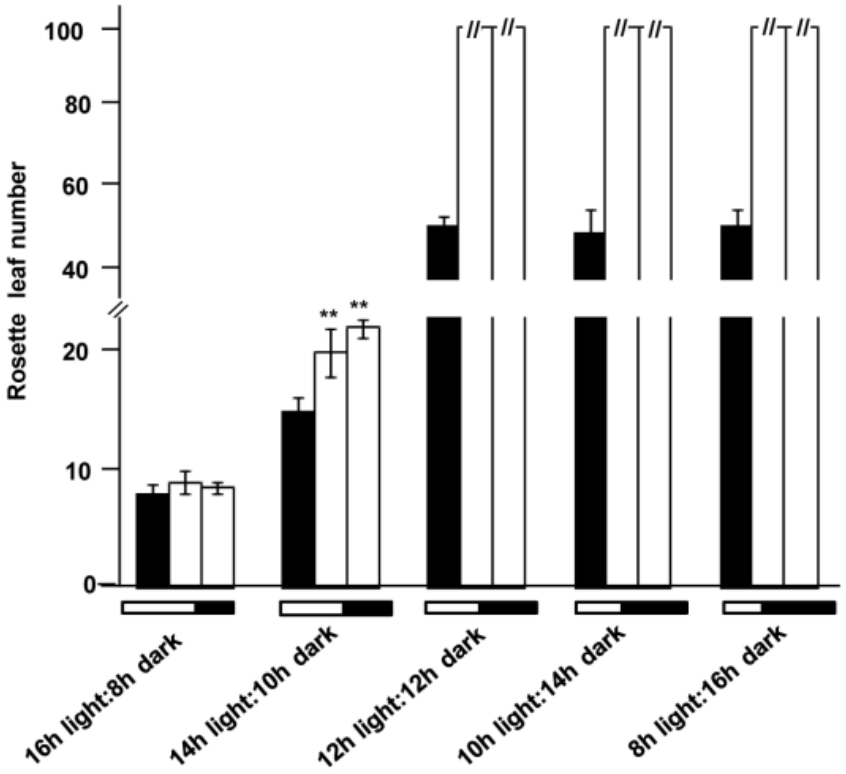

Figure 2.6: Effect of day-length on the flowering time of *bhlh93* mutant.

Wt and *bhlh93* mutants were grown under different photoperiods as indicated, and flowering time was measured using the number of rosette leaves at the time of flowering. **, indicates statistically significant difference with a p value $\leq$0.01. n>15

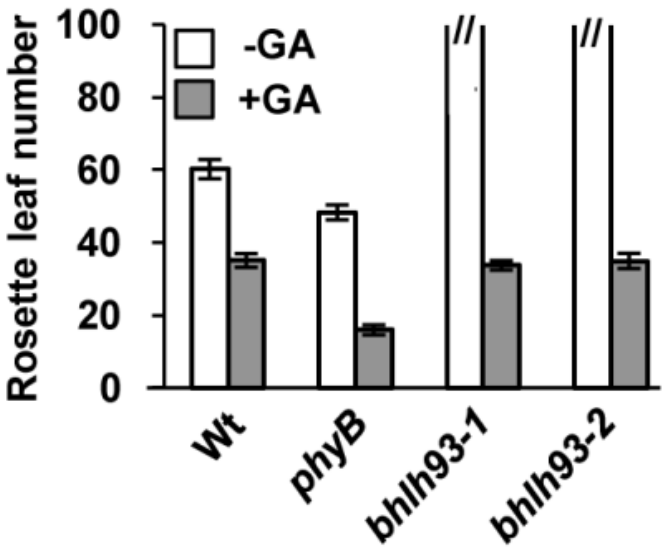

Figure 2.7: Exogenous application of gibberellin (GA_4) rescues the late flowering phenotypes of *bhlh93* mutant under SD conditions.

Bar-graph showing the number of rosette leaves produced by various genotypes at the time of flowering. Plants were grown under SD (8h light/16h dark) conditions with and without GA application. n>15

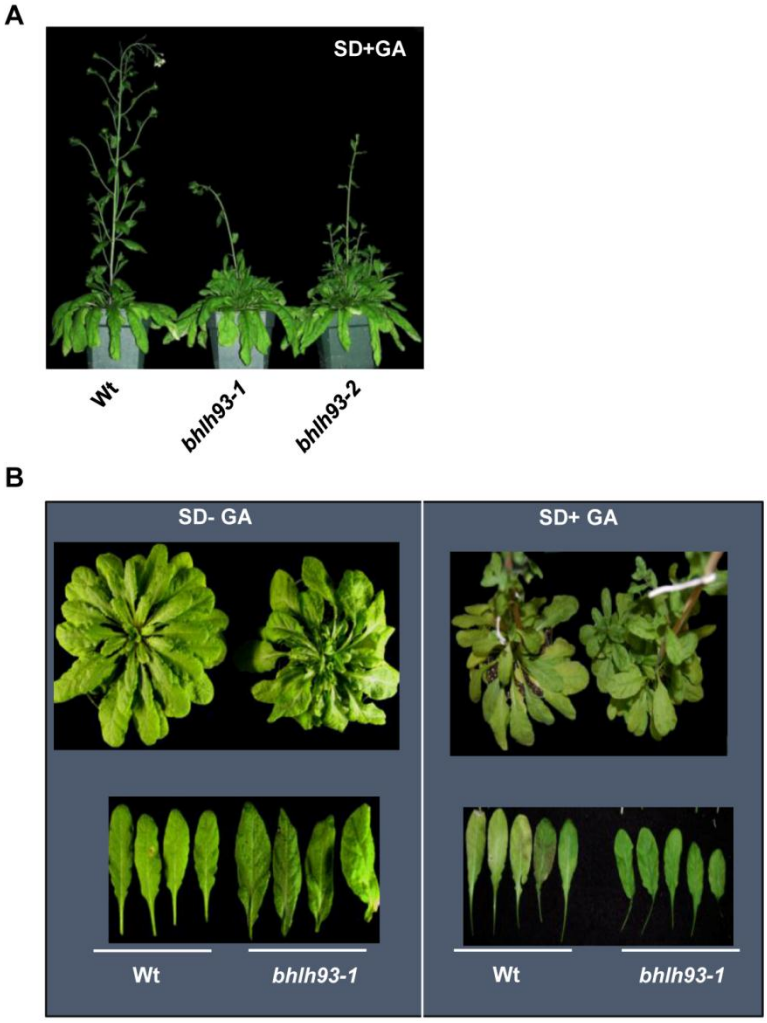

Figure 2.8: Exogenous application of gibberellin (GA_4) rescues the late flowering and leaf morphology phenotypes of *bhlh93* mutant under SD conditions.

A) Photographs of wt and two alleles of *bhlh93* mutant plants grown under SD conditions and treated with GA_4. B) Photographs showing rosette morphology and curly leaf phenotypes of wt and *bhlh93* mutants treated without (left) and with GA_4 (right). n>15

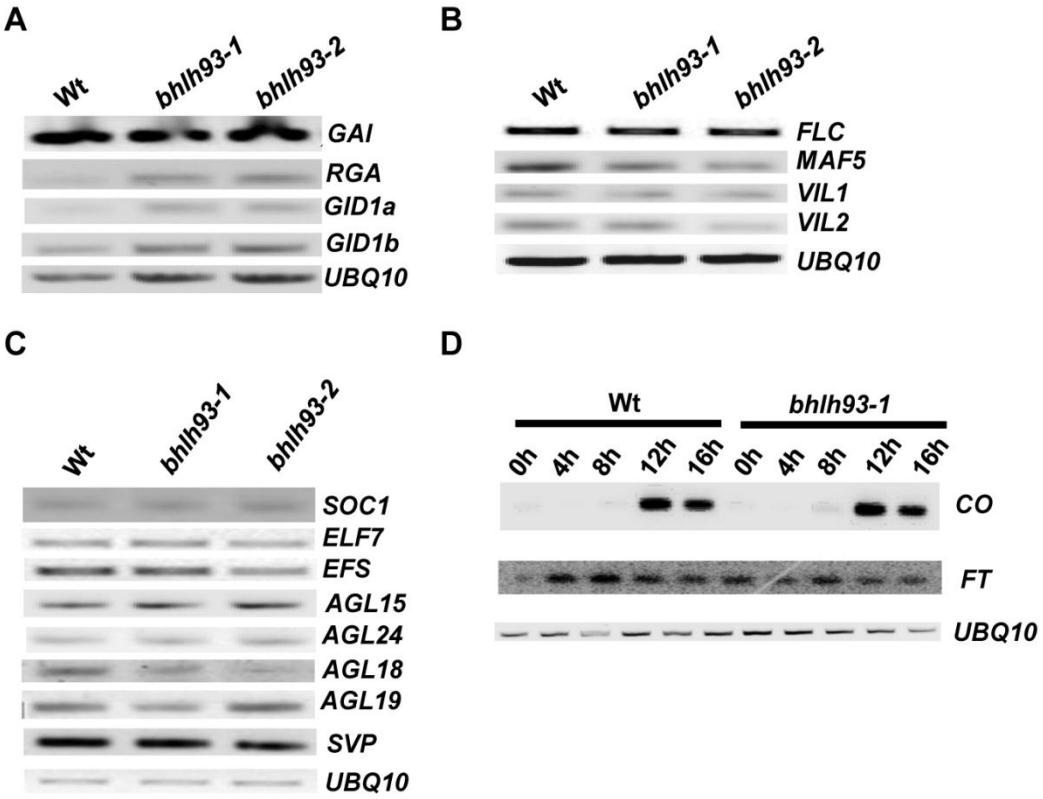

Figure 2.9: Expression of genes involved in GA signaling, vernalization and flowering time in wild type and *bhlh93* mutant seedlings.

A) Semi-quantitative RT-PCR for GA signaling genes in wild type and *bhlh93* mutant seedlings. B) Semi-quantitative RT-PCR for vernalization pathway genes in wild type and *bhlh93* mutant. C) Semi-quantitative RT-PCR for flowering time genes in wild type and *bhlh93* mutant seedlings. D) Semi-quantitative RT-PCR followed by Southern blot for flowering time genes in wild type and *bhlh93* mutant seedlings. Total RNA was isolated from 10 day-old seedlings grown under SD conditions. n=3

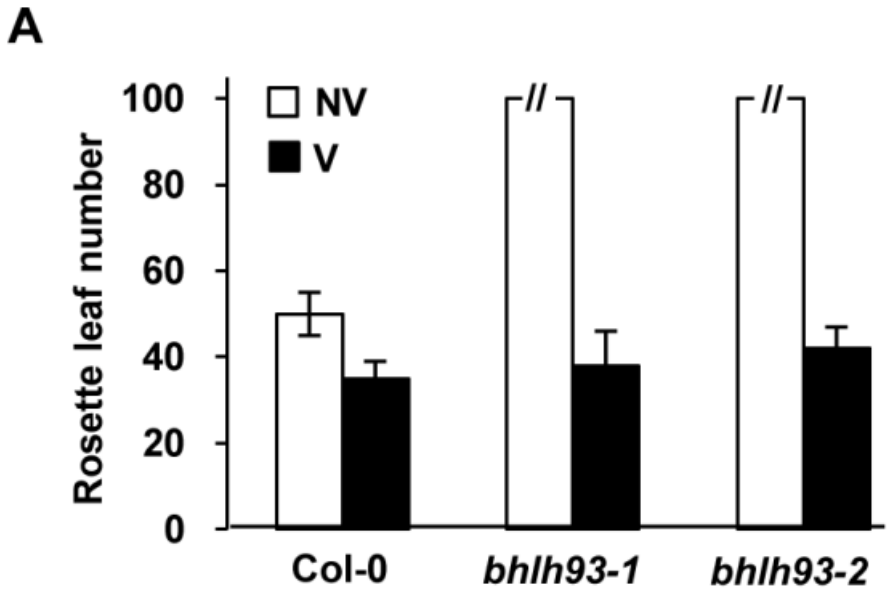

Figure 2.10: Flowering time phenotype for the wild type and *bhlh93* mutant in response to vernalization under SD conditions.

Bar-graphs showing average number of rosette leaves at bolting under SD conditions. Plants were vernalized for 56 days at 4^0C and then grown under SD (8h light/16h dark) conditions at 21^0C until bolting. n>15

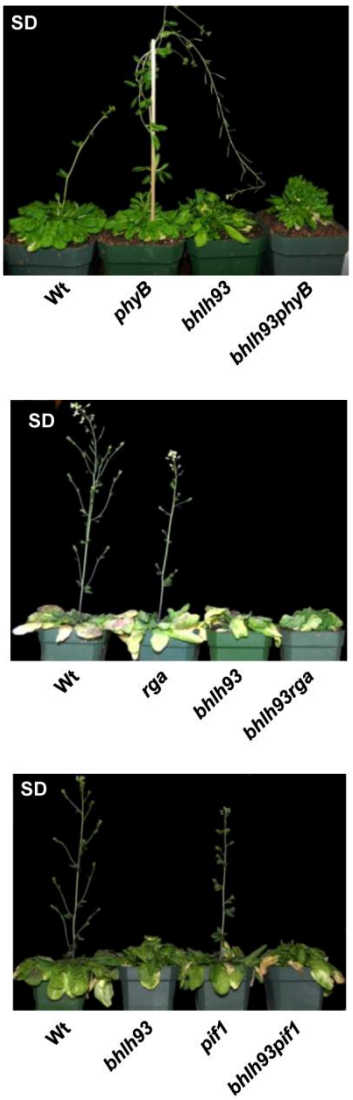

Figure 2.11: Double mutant phenotype of various genotypes.

Photographs showing *bhlh93* and various double mutant of *bhlh93* did not flower under SD conditions. n>15

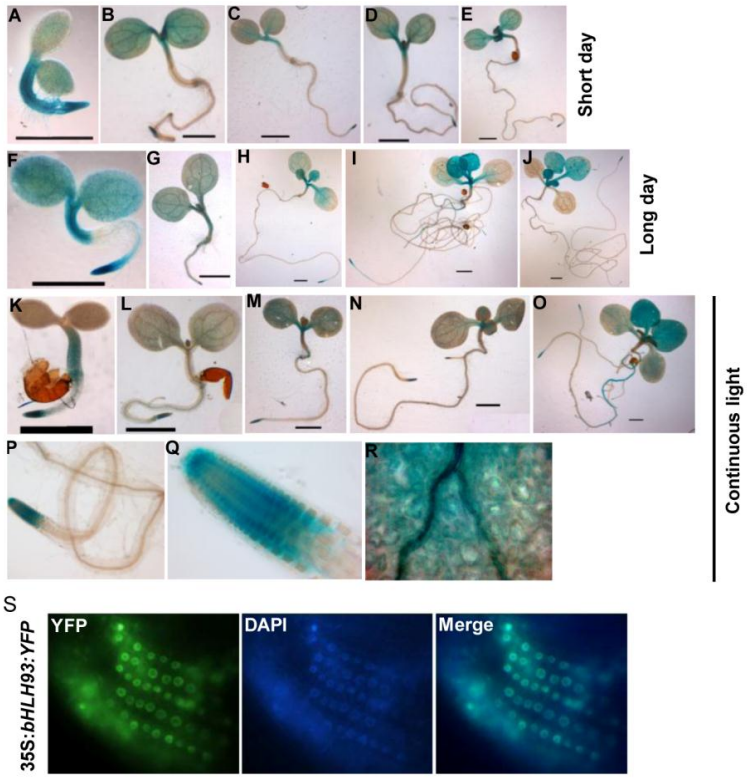

Figure 2.12: Tissue specific expression of *bHLH93* under SD (A-E), LD (F-J), and continuous light (K-R) conditions.

bHLH93 is expressed in the cotyledon, hypocotyl and root at 2 day-old seedlings grown under SD, LD and continuous light (A, F, and K). At day 4 and day 6 of growth, *bHLH93* is expressed in the cotyledon and hypocotyl (SD, LD), root (SD, LD and continuous light; B, G, and L). At days 8 and 10, *bHLH93* is expressed in the true leaves under LD and continuous light conditions. Higher magnification shows *bHLH93* is expressed mainly in root tips and veins in the cotyledons (P, Q and R). S) bHLH93 is localized to nucleus. Subcellular localization of bHLH93-YFP fusion protein in transgenic plants. Left panel shows the YFP fluorescence, the middle panel shows the DAPI staining of the nuclei, and right panel shows the merge between YFP and DAPI signals. n=3

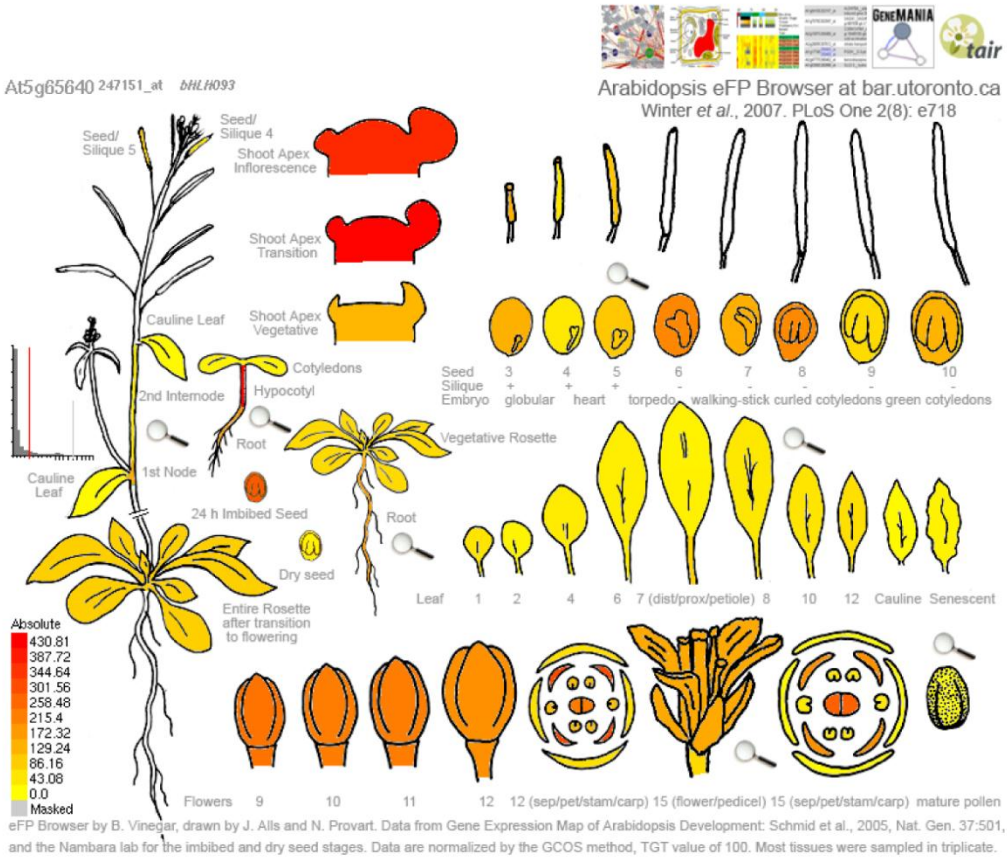

Figure 2.13: Developmental expression patterns for *bHLH93*.

Digital expression patterns for *bHLH93* in various tissues were obtained from eFP Browser web site (Winter et al., 2007).

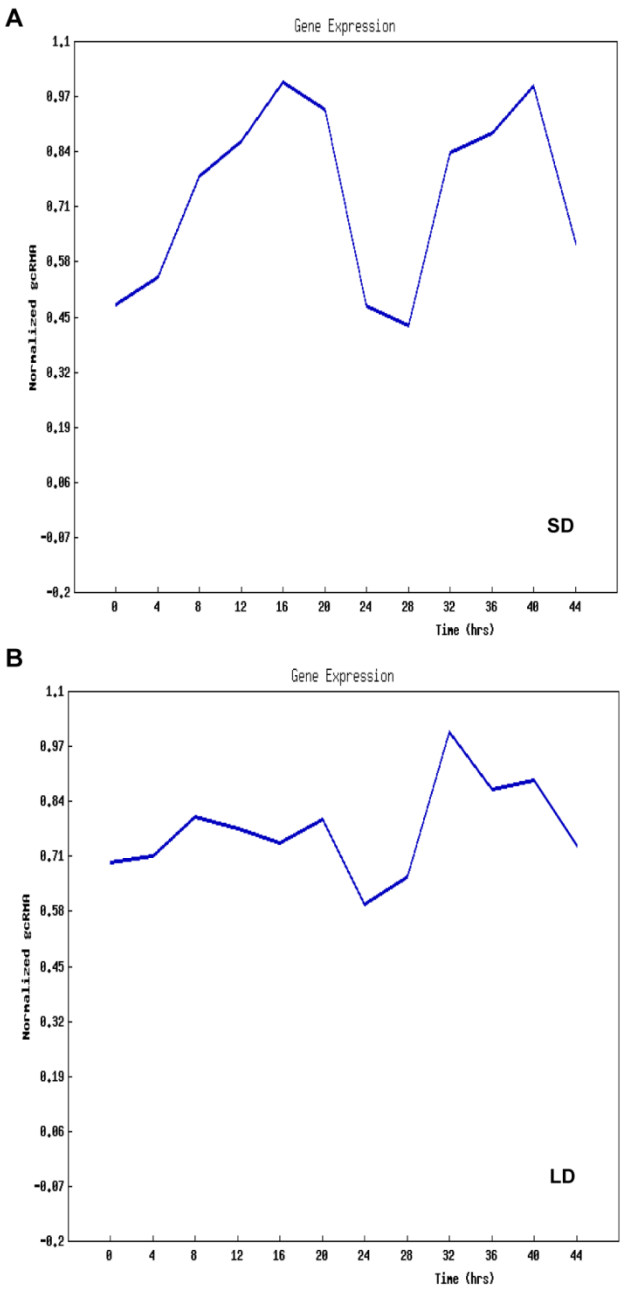

Figure 2.14: Diurnal expression of *bHLH93* under SD and LD conditions.

bHLH93 shows more robust diurnal regulation of expression in wild type plants under SD conditions compared to LD conditions. These data were obtained from publicly available data (Mockler et al., 2007).

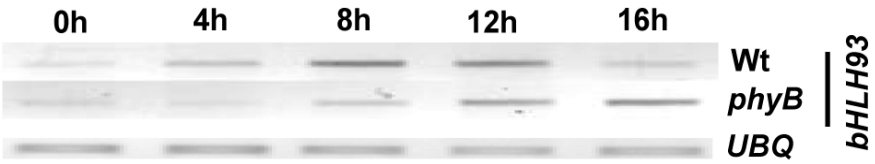

Figure 2.15: Expression of *bHLH93* under SD conditions.

bHLH93 shows diurnal regulation of expression in wild type plants. Expression peaks at 8-12h after the light is turned on. This peak of expression of *bHLH93* is shifted in *phyB* mutant under SD conditions. In *phyB* mutant, *bHLH93* expression peaks at 16h of day. n=3

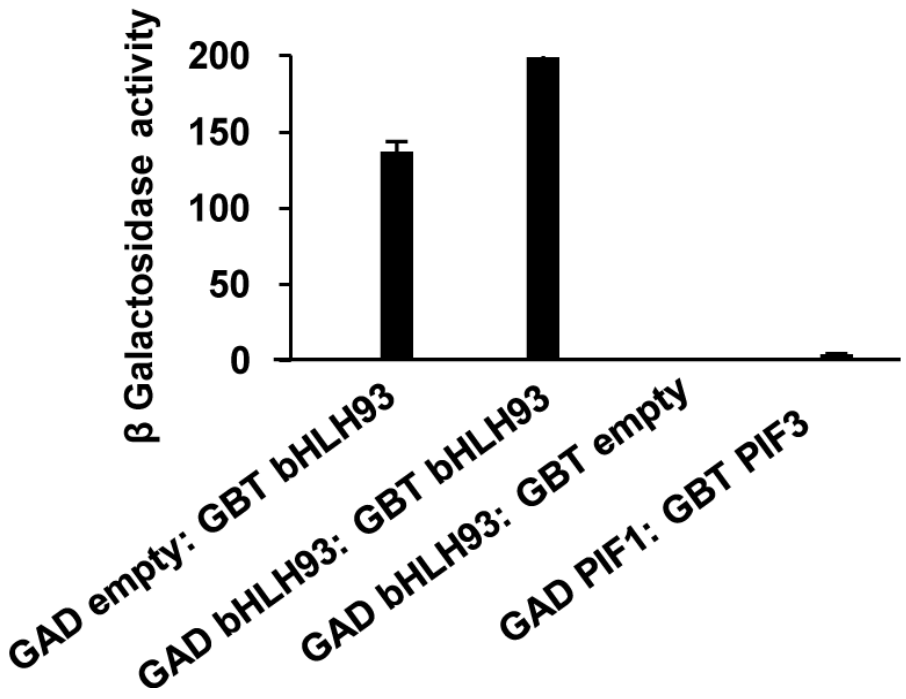

Figure 2.16: Homo-dimerization and transcriptional activation activity of bHLH93 in yeast two-hyrbrid assays.

β-Galactosidase assays were performed in triplicate and the data represent mean ± s.e.m. β-Galactosidase units are Miller units (M.U.). GAD, gal4 activation domain, and GBD, gal4 DNA binding domain.

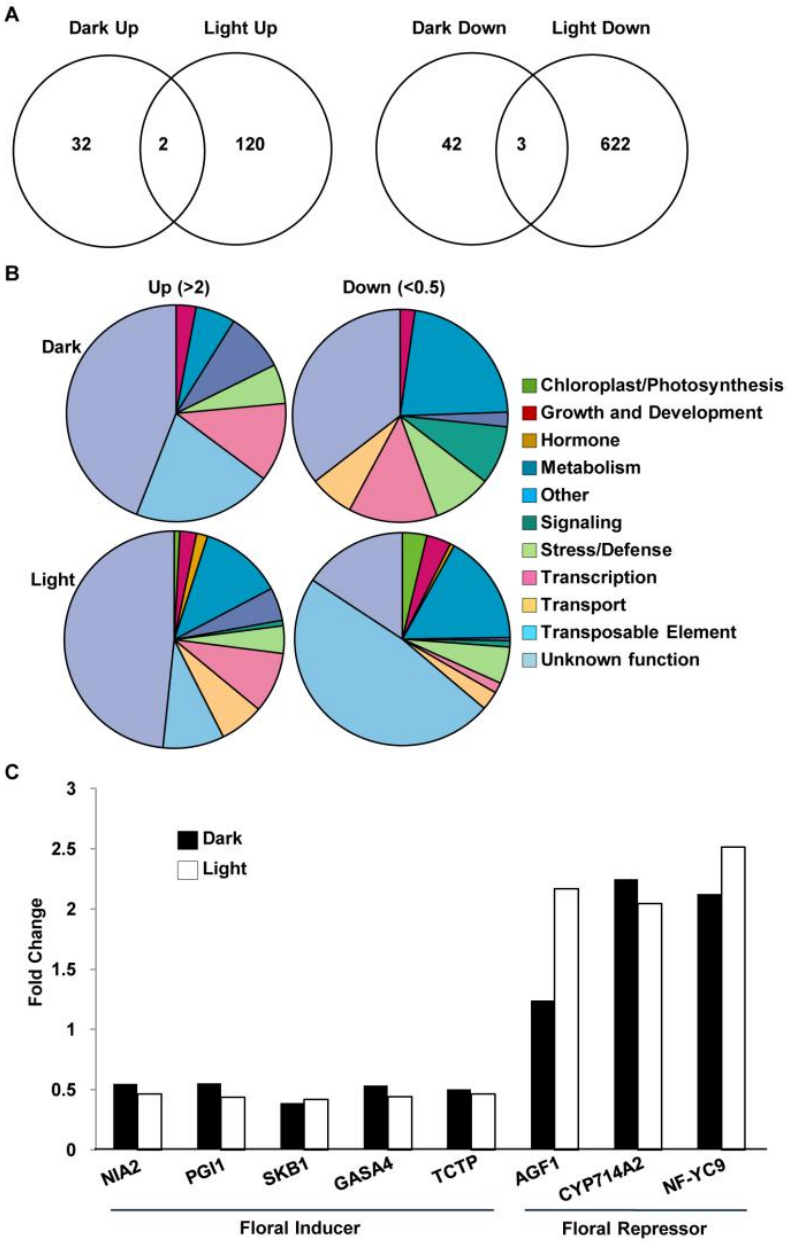
A
Dark Up
Light Up
Dark Down
Light Down
32
2
120
42
3
622
B
Up (>2)
Down (<0.5)
Dark
Light
Chloroplast/Photosynthesis
Growth and Development
Hormone
Metabolism
Other
Signaling
Stress/Defense
Transcription
Transport
Transposable Element
Unknown function
C
Dark
Light
Fold Change
3
2.5
2
1.5
1
0.5
0
NIA2
PGI1
SKB1
GASA4
TCTP
AGF1
CYP714A2
NF-YC9
Floral Inducer
Floral Repressor

Figure 2.17: Genome wide expression analyses in *bhlh93* mutant and wt seedlings.

A) Venn diagram showing the number differentially expressed genes in *bhlh93* mutant compared to wt seedlings in the dark and 4h after light is on. These differentially expressed genes are induced or repressed at least two-fold in *bhlh93* mutant in the dark and light with a q value $\leq$0.05. Seedlings were grown under SD conditions and dark samples were collected at the end of the dark period and the light samples were collected 4h after the light is on. B) Functional categorization of differentially regulated genes in *bhlh93* mutants relative to wild type controls. C). Bar-graph showing upregulation or downregulation of a set of genes implicated in either repression or promotion of flowering time, respectively, in *bhlh93* mutant compared to wt seedlings. These differentially expressed genes are induced or repressed at least two-fold in *bhlh93* mutant in dark and light with a q value $\leq$ 0.05. n=3

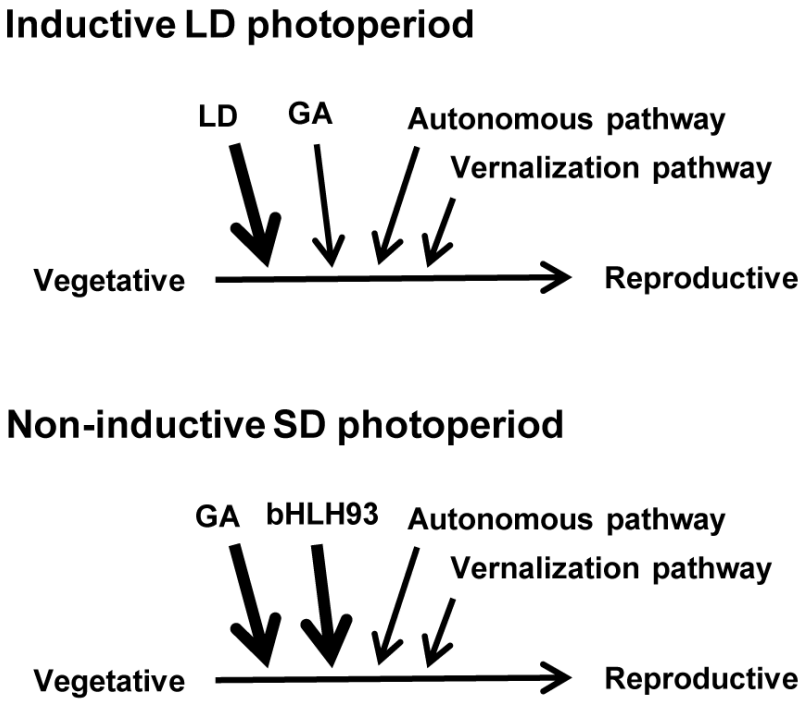

Figure2.18: A simplified model showing the role of bHLH93 in a photoperiod-dependent regulation of flowering time.

bHLH93 is not necessary for floral transition under inductive photoperiod (LD) conditions (top), where LD pathway plays a major role in regulating flowering time. Autonomous, GA and vernalization pathways play minor roles under LD conditions. In contrast, bHLH93 is essential for floral transition under non-inductive photoperiod (SD) conditions (bottom). GA also plays a major role, while autonomous and vernalization pathways play minor roles in promoting flowering time under non-inductive SD conditions.

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CHAPTER 3: Identification and functional characterization of target genes for bHLH93

ABSTRACT

Arabidopsis is a facultative long day (LD) plant where LD condition promotes flowering. *Arabidopsis* also flowers under short day (SD) conditions; however, flowering is delayed under these conditions. The molecular mechanism for this facultative behavior is still unknown. Recently, we have shown that *bhlh93* mutants never flowered under SD, but flowers similar to wt under LD conditions. Thus, *bHLH93* plays a pivotal role in promoting flowering time specifically under SD conditions. To understand the molecular mechanism by which bHLH93 regulates flowering time, we performed additional phenotypic characterization. Results showed that *bhlh93* mutant phenotypes appeared more prominently at later stages of development than at the seedling stage. Transfer of SD grown plants to LD conditions at various ages showed that *bhlh93* mutants lose competency to flower after 30 days of growth under SD. Vernalization treatment showed that *bhlh93* mutants require 8 weeks of vernalization treatments to promote flowering under SD conditions, suggesting that, although *bhlh93* is responding to vernalization, it is hyposensitive to vernalization treatments. Quantitative RT-PCR analyses from the meristem tissues of adult plants showed that *FLC* clade genes, especially *FLC* and *MAF5*, are upregulated in *bhlh93* mutant compared to wt controls. Chromatin immunoprecipitation assays showed that bHLH93 associates with *MAF5* promoter region containing E boxes. Gel-shift assays with E-box region from MAF5 promoter showed that bHLH93 can directly bind to *MAF5* promoter. These data suggest that bHLH93 directly targets *MAF5* and possibly other *FLC* clade genes to regulate flowering time specifically under SD conditions.

INTRODUCTION

Plants being sessile organisms do not have the ability to travel to a favorable environment. To avoid adverse conditions, plants have evolved genetic mechanisms to optimize growth and development. One mechanism to maximize reproductive success of the plant is to regulate transition from vegetative to reproductive phase. This transition, known as flowering, occurs when the shoot apical meristem (SAM) is converted to inflorescence meristem which is eventually modified into floral meristem. This switch from SAM to floral meristem is a tightly regulated process where several genetic pathways come together to induce flowering. The timing of flowering is fine tuned by other environmental and endogenous signals such as light, temperature, nutrients, hormones, etc.

In *Arabidopsis*, two floral integrator genes FLOWERING LOCUS T (FT) and SUPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) activate the downstream floral identity genes (e.g., *APETALA1, AP1* and *LEAFY, LFY*) to induce flowering (Michaels and Amasino, 1999; Blazquez and Weigel, 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000; Moon et al., 2003; Moon et al., 2005). *SOC1* and *FT* expression is regulated by four genetic pathways, namely, vernalization, autonomous, hormones, and photoperiod pathways.

Vernalization, or prolonged cold exposure, represses *FLOWERING LOCUS C* (*FLC)* and *FLC*-clade members including MADS-box transcription factors *FLOWERING LOCUS M / MADS AFFECTING FACTOR 1* (*MAF1*) and *MAF 2-5* to induce flowering time in *Arabidopsis* (Kim et al., 2009; Kim and Sung, 2010). Prior to vernalization, FLC represses expression of *FT* and *SOC1* in phloem and in the meristem and *FD* in the meristem (Searle et al., 2006). However, vernalization induces an epigenetic modification

at *FLC* and some *FLC* clade members locus via VERNALIZATION INSENSITIVE 3 (VIN3) (Kim et al., 2009). The repression of *FLC* and *FLC* clade members leads to activation of *FT* and *SOC1* that allow plants to flower after a long duration of cold exposure (Kim et al., 2009; Dennis et al., 2007; Sung et al., 2004; Henderson et al., 2004; Sung et al., 2006; Sheldon et al., 2009). *VIN3* belongs to a small gene family, known as the *VIN3/VERNALIZATION LIKE (VEL)* gene family (Sung et al., 2006; Greb et al., 2007). VEL gene family consists of *VIN3-LIKE 1* (*VIL1*)/*VERNALIZATION 5* (*VRN5*), *VIL2/VEL1, VIL3/VEL2,* and *VIL4/VEL3,* while *VIL4/VEL3* appears to be a pseudogene (Sung et al., 2006; Greb et al., 2007). In eukaryotes, Polycomb Repressive Complex 2 (PRC2) promotes histone modification during epigenetic regulation. A member of PRC2, ENHANCER OF ZESTE [E(Z)] adds methyl groups at H3K27 residues (H3K27me3)(Muller et al., 2002). In *Arabidopsis*, VRN2, VIN3 and VRN5/VIL1 appear to form a repressive complex similar PRC2 and this complex is required for histone modification at *FLC* locus (Wood et al., 2006; De Lucia et al., 2008). *Arabidopsis* has at least two homologs of E(Z) called CURLY LEAF (CLF) and SWINGER (SWN) in the VIN3 and VIL1/VRN5 complex (Wood et al., 2006; De Lucia et al., 2008). Thus, only epigenetic regulation of *FLC* and *FLC*-clade members has been shown in flowering time in *Arabidopsis*.

Photoperiod (day length) also regulates flowering time in *Arabidopsis* (Fornara et al., 2010). Long day (LD, 16h light/8h dark) acts as an inductive photoperiod in *Arabidopsis* and promotes flowering. However, flowering is delayed under non-inductive SD (8h light/16h dark) photoperiod. LD pathway functions via *CONSTANS (CO)* gene and *CO* is regulated in part by two antagonistic groups of genes: activators *FLAVIN-BINDING, KELCH REPEAT, AND F-BOX 1(FKF1), GIGANTEA (GI),* and repressors *ELF3, CYCLING DOF FACTOR1 (CDF1),* and *RED AND FAR-RED INSENSITIVE 2*

(RFI2) (Fowler et al., 1999; Nelson et al., 2000; Covington et al., 2001; Sua´rez-Lo´pez et al., 2001; Imaizumi et al., 2005; Chen and Ni, 2006). Stable CO protein promotes expression of *FT*. FT associates with FD to activate expression of *SOC1* and downstream floral identity genes such as *AP1* and *LFY* to promote flowering (Abe et al., 2005; Michaels et al., 2005; Wigge et al., 2005; Yoo et al., 2005).

Unlike LD pathway, non-inductive SD pathway is poorly understood. Recently we identified a transcription factor, bHLH93 that is necessary to induce flowering specifically under SD conditions. *bhlh93* mutants do not flower under SD, but flower like wt in LD. However, how bHLH93 regulates flowering only under SD conditions is still unknown. Here we show that bHLH93 mainly functions at the adult stage and controls adult vegetative to adult reproductive phase transition. Thus we mainly focused on adult stage in this study. We show that *bhlh93* mutants lose floral competence at 30 days of growth in SD and *bhlh93* mutants need prolonged vernalization treatment to promote flowering in SD. Recently, VIL1-2 has been shown to promote flowering through chromatin modification of *MAF1* and *MAF5* genes, respectively under SD conditions (Sung et al., 2006; Kim and Sung, 2010). *vil1* and *vil2* mutants flower late under SD specifically, but *bhlh93* mutants do not flower at all in SD. Here we have identified *MAF5* and *FLC* as putative targets of bHLH93 to promote flowering. We describe a novel mechanism of *MAF5* regulation by a transcription factor. bHLH93 directly binds *MAF5* promoter region containing consensus E-box sequence. This is the first report of *MAF5* gene regulation directly by a transcription factor other than epigenetic modification.

MATERIALS AND METHODS

Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24-h light or long day (LD, 16h light, 120 $\mu molm^{-2}s^{-1}$ and 8h dark), or short day (SD, 8h light, 200 $\mu molm^{-2}s^{-1}$ and 16h dark), at $21^0C \pm 0.5^0C$. Light fluence rates were measured using a spectroradiometer (model EPP2000; StellarNet Inc., Tampa, FL) as described (Shen et al., 2005). T-DNA-tagged *bhlh93* seeds from SALK collection were obtained from the *Arabidopsis* Biological Resource Center (Alonso et al., 2003). Seeds were surface sterilized and plated on Murashige and Skoog growth medium (GM) containing 0.9% agar without Suc (GM - Suc) as described (Shen et al., 2005). After 4 days of stratification at 4^0C, seeds were exposed to SD or LD or continuous white light conditions.

Loss of floral competence

bhlh93 and wt plants were grown under SD conditions and then transferred to continuous light conditions. Flowering time was noted at the time of bolting.

Vernalization treatment

After stratification in dark for 4 days, seeds were exposed to SD (8 h of light and 16 h dark) for 7 days for germination at $21^0C \pm 0.5^0C$. Seedlings were vernalized for six, eight and ten weeks at 4°C under SD. Vernalized seedlings continued to grow in SD at $21^0C \pm 0.5^0C$ until bolting. Flowering time was quantified in both non-vernalized and vernalized plants using rosette leaf number and days to flower.

Quantitative RT-PCR analyses

Total RNA was isolated from meristems of 54 day-old *bhlh93-1* mutants and wt Col-0 plants grown under SD using the RNeasy plant mini kit (Qiagen, Valencia, CA).

For reverse transcription reactions, total RNA was treated with DNase I to remove genomic DNA. One µg of total RNA was reverse transcribed using the RT-PCR kit (Invitrogen Inc., Carlsbad, CA), and first-strand cDNA was used as a template for PCR amplification. For quantitative RT-PCR, 20 µL cDNA was diluted to 40 µL with water and 1 µL of diluted cDNA was used for PCR amplification using gene-specific primers. The *PP2A* fragment was used as a control to normalize the amount of cDNA used. The qRT-PCR primer sets are shown in Table 3.1.

Purification of bHLH93-His recombinant protein from *E.coli.*

bHLH93 cDNA was amplified using primers listed in Table 3.1. bHLH93 cDNA was cloned into pCR®-Blunt II-TOPO® Vector (Zero Blunt® TOPO® PCR Cloning Kit, Invitrogen). bHLH93 was cut with EcoR1 enzyme from bHLH93-TOPO clone and ligated to EcoR1-cut pCold vector (Takara Bio Inc.). bHLH93-pCold clone was transformed into SoluBL21 competent cells according to user manual (Genlantis, Inc.). bHLH93-His protein in SoluBL21 was induced with 1mM IPTG in M9 minimal media at 26^{o}C overnight. Recombinant bHLH93-His protein was purified using Ni-NTA resin according to user manual (Qiagen).

Gel shift Assay

DNA gel-shift assays were performed as described (Huq and Quail, 2002; Moon et al., 2008). EcoR1-cut bHLH93 from bHLH93-TOPO clone (described above) was ligated to EcoR1-cut pTnT vector (Promega). bHLH93 protein was synthesized according to user manual (Promega). A 41-bp MAF5 promoter fragment containing a E-box motif was labeled with ^{32}P-dCTP. The binding conditions and gel compositions were as described (Huq and Quail, 2002).

Chromatin Immunoprecipitation Assay

ChIP assays were performed as described (Gendrel et al., 2002), except meristems from 54-day-old SD grown *pbHLH93:bHLH93-GUS* (Sharma et al., 2011) and wt plants were harvested at end of dark period. Tissue was vacuum-infiltrated with 1% formaldehyde for 1 h at 4°C, and cross-linking was quenched by vacuum infiltration with 0.125 M glycine for 15 min. Antibody against GUS (Invitrogen Inc., Carlsbad, CA) was used for IP.

Double mutant analysis

bhlh93-2 and *flc-3* mutants were artificially crossed to get *bhlh93-2flc-1* double mutants. Double mutant plants were PCR screened for homozygous genotype using primers listed in Table 3.1. Flowering phenotype of *bhlh93-2flc-1*homozygous mutant was quantified using both number of days to flower and number of rosette leaves formed at the time of flowering. *bhlh93maf5* double mutants were screened using primers listed in Table 3.1.

RESULTS

bhlh93 show mutant phenotype later in development

bhlh93 mutants have severe mutant phenotype, however we observed that this mutant phenotype appears late in development (Fig 1). Under SD, the *bhlh93* mutants looks similar to wt until 40 days. But around 55 days in SD, *bhlh93* mutants start showing curled leaves and the meristem is different than Wt. Eventually after 70 days in SD, *bhlh93* mutant shows deformed meristem and prominent curled leaves (Fig 3.1). This suggests that bHLH93 functions later in development of *Arabidopsis*.

bhlh93 mutants lose competence to flower after 30 days in SD

As mentioned above, *bhlh93* mutants show a mutant phenotype later in development and fail to flower in SD. Thus we were interested in identifying the developmental stage when *bhlh93* mutants lose competence to flower. We grew wt and *bhlh93* mutants under SD conditions and then transferred *bhlh93* and wt from SD to continuous light. Both wt and *bhlh93* mutants flowered similar to wt plants when 0-30 day-old plants are transferred from SD to continuous light (data not shown). However, *bhlh93* failed to flower after 30 days of growth under SD conditions, while wt plants flower normally even after 60 days of growth under SD conditions (Fig 3.2). These data suggest that *bhlh93* mutants lose competence to flower after 30 days of growth under SD.

bhlh93 mutants respond to saturating vernalization treatment

Because *bhlh93* mutants show age dependent flowering phenotype, we tested the effect of increasing amount of vernalization treatment on *bhlh93* flowering time. We treated *bhlh93* and wt plants for four, six, and eight weeks at 4^{o}C and then transferred them to SD conditions. *bhlh93* mutants failed to flower after four and six weeks of vernalization; however, *bhlh93* flowered like wt after eight weeks of vernalization (Fig 3.3). These data suggest that *bhlh93* mutants might be hyposensitive to vernalization treatment and may require longer vernalization to induce flowering under SD conditions.

Floral repressors *MAF5* and *FLC* are upregulated in *bhlh93* mutants

Vernalization treatment causes epigenetic repression of floral repressors *FLC* and *FLC*-clade members including *MAF1-5*. We examined the expression profiles of these floral repressors in *bhlh93* and wt plants. Previously we investigated possible target genes of bHLH93 at the juvenile stage and we did not find any strong candidate. Thus, we focused on adult stage to identify bHLH93 target genes in this study. We harvested

meristems from different developmental stages such as 30 and 54 day-old *bhlh93* and wt plants grown in SD (Fig 3.4 A and B). RNA from these meristems was used to perform a quantitative RT PCR to investigate expression levels of floral repressors in *bhlh93*. Our results show that in *bhlh93, MAF5* is two- and four-fold upregulated in 30-d- and 54-d-old plants, respectively (Fig 3.4 A, B). This result suggests an increasing activation of *MAF5* during development in *bhlh93*. The linear upregulation of *MAF5* in *bhlh93* correlates with the timing of loss of floral competence in *bhlh93* in SD. The higher levels of *MAF5* in *bhlh93* plants suggest *MAF5* is directly or indirectly regulated by bHLH93 in SD.

We examined relative expression of *bHLH93* and *MAF5* in wt plants grown under SD. The results show that the expression of *bHLH93* increases with developmental stage from 10d to 68d in SD (Fig 3.4 C). However, *MAF5* expression is maintained low throughout the development as compared to bHLH93. We see similar repression of *MAF5* corresponding to higher *bHLH93* in publicly available expression data (Fig 3.5). This result is consistent with the hypothesis that bHLH93 represses *MAF5* expression in SD.

Other than *MAF5*, *FLC* and two other FLC-clade members namely *FLM/MAF1* and *MAF2* are also upregulated in *bhlh93*. *FLC* and *FLM/MAF1* are ~1.5-fold upregulated in both the dark and light in *bhlh93* whereas *MAF2* is ~1.5-fold upregulated in dark only (Fig 3.4 A). These results suggest *FLC* and *MAF1-2* are also possible targets of bHLH93.

bHLH93 binds to *MAF5* promoter in vivo (ChIP)

bHLH transcription factors have been shown to regulate expression of target genes by binding to a consensus promoter element (CANNTG) called E-box. We

examined *MAF5* promoter region and found three E-boxes. Two of these E-boxes at the 5' of promoter are very close to each other and the third E-box is closer to transcription start site of *MAF5* (Fig 3.6 A). bHLH93 is a transcription factor and *MAF5* is a main candidate target of bHLH93. Thus we were interested to see if bHLH93 directly or indirectly regulates *MAF5* expression. We performed a chromatin immunoprecipitation (ChIP) assay using *pbHLH93:bHLH93-GUS* transgenic plants (Sharma et al, 2011). We harvested meristems from different developmental stages like 54-d-old *pbHLH93:bHLH93-GUS* and wt plants grown in SD. After immunoprecipitation of protein–DNA complexes using antibody to GUS, enriched DNA sequences were amplified using primers to the promoter regions of the *MAF5*. ChIP assay results show that the *MAF5* Ebox2 region was amplified from the immunoprecipitation (IP) fraction of *pbHLH93:bHLH93-GUS* meristems but not the upstream Ebox1 (Fig 3.6 B). This data suggests bHLH93 associates with *MAF5* promoter element at Ebox2 site *in vivo* and regulates *MAF5* expression.

bHLH93 binds to the E-boxes present in *MAF5* promoter in vitro

To determine whether bHLH93 directly binds the E box 2 within *MAF5* promoter, a gel-shift assay was performed. We expressed bHLH93 protein in TnT expression system and used MAF5 Ebox2 probe to examine DNA binding. Our results show that bHLH93 binds the labeled *MAF5* Ebox2 promoter, which can be competed with wt cold probe (Fig 3.7). Although, further studies are necessary to examine the specificity of this binding using mutant cold probes, these preliminary data suggest that bHLH93 directly binds to the E-box region of MAF5 promoter..

***bhlh93* is epistatic to *flc* (double mutant phenotype)**

FLC is another possible target of bHLH93, and we tested genetic interaction between *bhlh93* and *flc* using double mutant analyses. We created *bhlh93flc* double mutants and compared their flowering phenotype with single mutants under SD conditions. *bhlh93flc* double mutants did not flower like *bhlh93* single mutant in SD (data not shown). This suggests *bHLH93* is epistatic to *FLC*. We are creating a double mutant between *bhlh93* and *maf5*, however bHLH93 and MAF5 are only 250kb apart on chromosome 5. We have not been successful in obtaining *bhlh93maf5*, though *bhlh93-/-maf5-/+* plants show *bhlh93* mutant phenotype under SD. On the other hand, *bhlh93-/+maf5-/-* show *maf5* phenotype under SD (data not shown). These data suggest that multiple FLC clade mutants in *bhlh93* background might be necessary to suppress *bhlh93* phenotype under SD conditions.

Purification of bHLH93-His recombinant protein from *E coli*.

We cloned bHLH93 cDNA into pCold vector (6 X His protein tag at the N-terminus) using EcoR1 enzyme. bHLH93-pCold clone was transformed in SoluBL21 competent cells and His-bHLH93 protein was induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG). A strong induction band is seen in SDS-PAGE gel stained with Coomasie Blue (Fig 3.8A). His-bHLH93 protein was purified using Ni-NTA resin and SDS-PAGE gel shows purified His-bHLH93 protein (Fig 3.8B). This recombinant protein will be used for future studies.

DISCUSSION

In *Arabidopsis*, vernalization signal is sensed at the meristem and leads to activation of *VIN3*. *VIN3*, in turn, induces an epigenetic modification of *FLC*. FLC belongs to MAF family of transcription factors including FLM/MAF1 and MAF2-5.

During epigenetic modification, *FLC* chromatin undergoes distinct histone modification such as H3K9 and H3K27 di- and tri-methylation, H4R3sme2, histone deacetylation, and H3K4 demethylation (Bastow et al., 2004; Finnegan and Dennis, 2007; Greb et al., 2007; Schmitz et al., 2008; Sung and Amasino, 2004; Sung et al., 2006). Until now, *FLC* and *MAFs* have been shown to be regulated via epigenetic modifications only. Here we describe an additional mechanism of regulation to repress floral repressors to promote flowering in SD. bHLH93, a transcription factor, directly represses *MAF5* and possibly other *FLC* clade gene expression in SD to promote flowering in SD.

The life cycle of flowering plants is characterized by three distinct phases: juvenile, adult vegetative, and adult reproductive phase. We recently described a novel mutant *bhlh93* that does not flower in SD. We noticed that *bhlh93* starts showing a mutant phenotype later in the development. *bhlh93* mutants show the same morphology as wt until 40 days in SD, and the mutant phenotype is prominent only after 70 days in SD (Fig 3.1). Apparently, *bHLH93* regulates adult vegetative to adult reproductive phase transition and has little or no role in juvenile-to-adult vegetative phase transition. *bhlh93* mutants undergo normal juvenile to adult vegetative phase of growth as observed by leaf morphology (Fig 3.1). We also observed that *bhlh93* loses competence to flower after 30 days in SD (Fig 3.2). Before 30d, *bhlh93* mutants can still flower from primary meristem after being transferred to continuous light chamber. Thus *bhlh93* shows developmental-specific regulation of flowering time and this result suggests that bHLH93 has a prominent role at later stage of development.

Vernalization promotes flowering in *Arabidopsis*. This prompted us to look at the effect of increasing vernalization treatment. However *bhlh93* mutants did not flower with four and six weeks of vernalization treatment and flowered like wt following eight weeks of vernalization (Fig 3.3). This result suggests that *bhlh93* is hyposensitive to

vernalization and *bhlh93* mutants need saturating cold treatment to induce flowering in SD. Previous reports have shown vernalization epigenetically represses FLC and FLC-clade members. Thus we tested expression of floral repressor genes involved in the vernalization pathway. We observed *MAF5* was two-fold higher in *bhlh93* and *FLC* showed 1.5-fold higher expression in *bhlh93* in 30-d-old plants grown under SD (Fig 3.4 A). *MAF5* expression is four fold in 54d old *bhlh93* plants in SD (Fig 4B). Relative expression profile shows in wt plants, *bHLH93* is highly expressed and *MAF5* expression is maintained low in SD (Fig 3.4 C, 3.5). Overexpression of *MAF5* and *FLC* in *bhlh93* background correlates with the *bhlh93* late flowering phenotype in SD. These results suggest *MAF5* is a major target of bHLH93 in regulating flowering time, and *FLC* is also a possible target of bHLH93.

This raised an intriguing question as how bHLH93 regulates *MAF5* expression. Recent reports show that a Plant Homeo Domain finger-containing protein, VIN3-LIKE 2 (VIL2) regulates *MAF5* expression by maintaining the epigenetically repressed state of *MAF5* (Kim et al, 2010). Thus, we investigated regulation of *MAF5* by bHLH93. bHLH93 belongs to basic-helix-loop-helix (bHLH) family of transcription factors. Many bHLH proteins have been shown to directly bind to their target genes to regulate transcription. In-vivo ChIP assay showed that bHLH93 associates with *MAF5* promoter region Ebox closer to transcription start site (Fig 3.6). In-vitro DNA binding assay showed that bHLH93 binds to *MAF5* Ebox 2, and this binding is competed by the wt MAF5 promoter fragment (Fig 3.7). This result suggests that bHLH93 binds to the MAF5 promoter. This study is the first report of a new mechanism of regulation of floral repressor *MAF5* suggesting fine-tuning of flowering in *Arabidopsis* by independent mechanisms.

Double mutant studies with *bHLH93* and *FLC* shows *bhlh93flc* homozygous double mutants do not flower in SD, a phenotype similar to *bhlh93* single mutant. This suggests *bHLH93* is epistatic to *FLC* in flowering time pathway. We have not been successful in making *bhlh93maf5* double mutants. *bHLH93* and *MAF5* genes are 250MB apart on chromosome 5. However, *bhlh93* homozygous and *maf5* heterozygous double mutant shows same late flowering phenotype as *bhlh93* single mutant. The opposite *maf5* homozygous and *bhlh93* heterozygous double mutants flower same as wt in SD. We have previously shown that the *bhlh93* heterozygous mutant flowers like wt in SD (Sharma et al, unpublished data). Further investigation is needed to confirm the double mutant phenotype. Together, these data suggest a novel regulation mechanism of floral repressors by a bHLH transcription factor.

Table 3.1: Primer sequences used in experiments described in the text.

Gene	Forward	Reverse
For Genotyping		
bhlh93-1	TTTTCGATGGACGAATCTGTC	TACTGATTTTTGGGACGATGG
bhlh93-2	CAGAGGTTCGTTCGCATTAAG	TTAATGGCGGATTTGATCATC
T-DNA (*bHLH93*)	GCGTGGACCGCTTGCTGCAACT (LBb1)	CTCCTAATATCGATGTCCTGTC
flc	TATCGCCGGAGGAGAAGC	TAGAAAGAAATAAAGCGAGAAA
maf5	CAATAGATCACGGACATCACG	CCATGCTGGAGAGAGATGAAG
For qRT-PCR		
bHLH93	CTT GTT CTG AGG GAG CTG AGC	CAGCTTCCACCATAACCTGCG
MAF5	GGTCTCATGGAGAAAGCTCGT	TTGTAGAGTTTGCCGGTGGAA
FLC	GCCAAGAAGACCGAACTCATGTTGA	CAACCGCCGATTTAAGGTGGCTA
MAF1/FLM	CCACACAAGGAGTTACTAGAAACAGTCC	GCCAGAACCTGGTTCTCTTCTCTCAG
MAF2	TCTCTGGAGGAACAGCTCGAGACT	GAGCAGCGGAAGAGTCTCCCGTA
PP2A	TATCGGATGACGATTCTTCGTGCAG	GCTTGGTCGACTATCGGAATGAGAG
For Cloning		
bHLH93 cDNA	CCTGTCGACTTACAAGCAGCTTCCACCATAAC	AGAGAATTCATGGAACTGTCGACTCAAATG

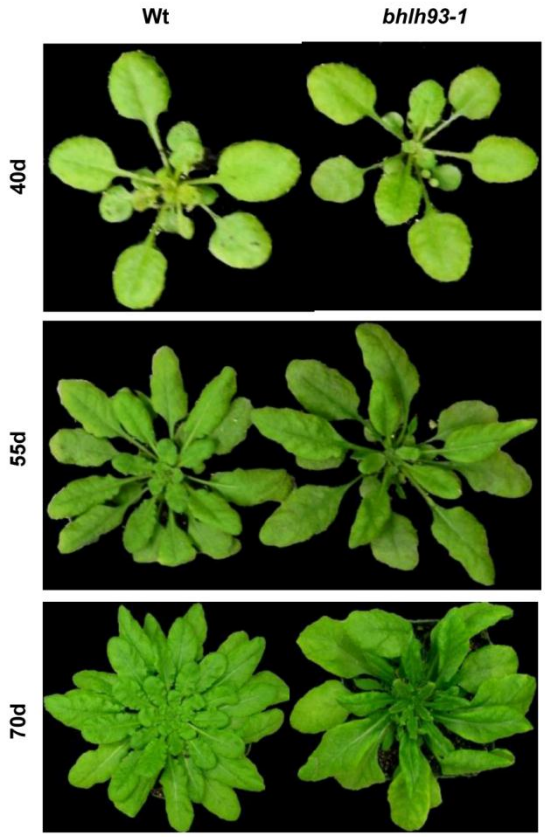

Figure 3.1: Adult phenotypes of *bhh93* mutants in SD.

bhlh93 and wt plants were grown under 8h light/16h dark SD photoperiod and visual phenotype was monitored throughout development. Digital images were taken to document the phenotype. *bhlh93* mutant starts developing mutant phenotypes only after 55d of growth under SD conditions. n>15

Figure 3.2: Flowering phenotype of wt and *bhlh93* transferred from SD to continuous light.

bhlh93 and wt plants were grown under SD conditions (8h light/16h dark) at 21^{o}C and plants of each genotype were transferred to continuous light every week. Flowering time was noted at the time of bolting. *bhlh93* mutants lose competence to flower after 30 days of growth in SD. n=2

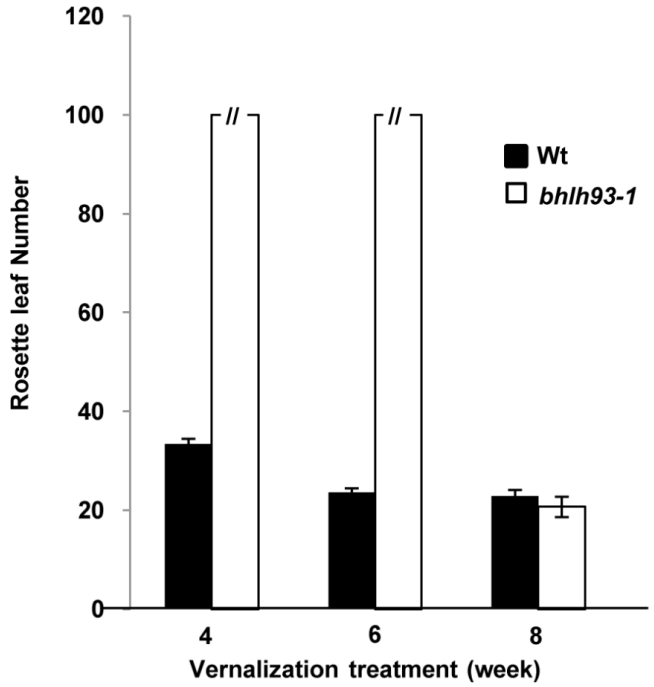

Figure 3.3: Flowering phenotype of wt and *bhlh93* after vernalization treatment.

bhlh93 and wt plants were grown under SD for 7 days to induce germination. Plants were vernalized at 4^{o}C (8h light/ 16h dark) for 4, 6 and 8 weeks and transferred back to SD at 21^{o}C. Flowering time was noted at the time of bolting. *bhlh93* mutants are hyposensitive to vernalization treatment and require at least 8 weeks of vernalization to induce flowering in SD. n>15

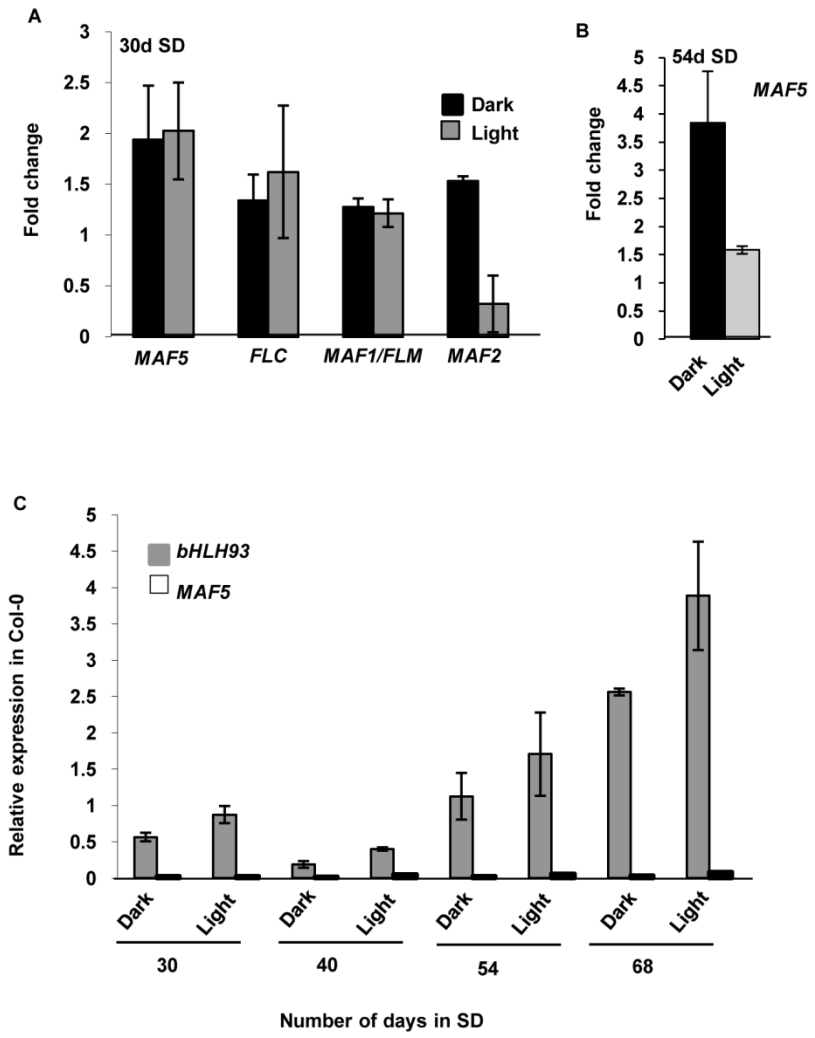

Figure 3.4: Expression of genes involved in floral repression.

A) qPCR of *MAF5, FLC, MAF1/FLM,* and *MAF2* after 30 days in SD. *bhlh93* and wt plants were grown under SD for 30 days and meristem was harvested at the end of dark period and 4 hours after light was turned on. B) qPCR of *MAF5* after 54 days in SD. *bhlh93* and wt plants were grown under SD for 54 days and meristem was harvested at the end of dark period and 4 hours after light was turned on. C) qPCR of *MAF5* and *bHLH93* at 30, 40, 54, and 68 days in SD. *bhlh93* and wt plants were grown under SD for 30, 40, 54, and 68 days and meristem was harvested at the end of dark period and 4 hours after light was turned on. n=3

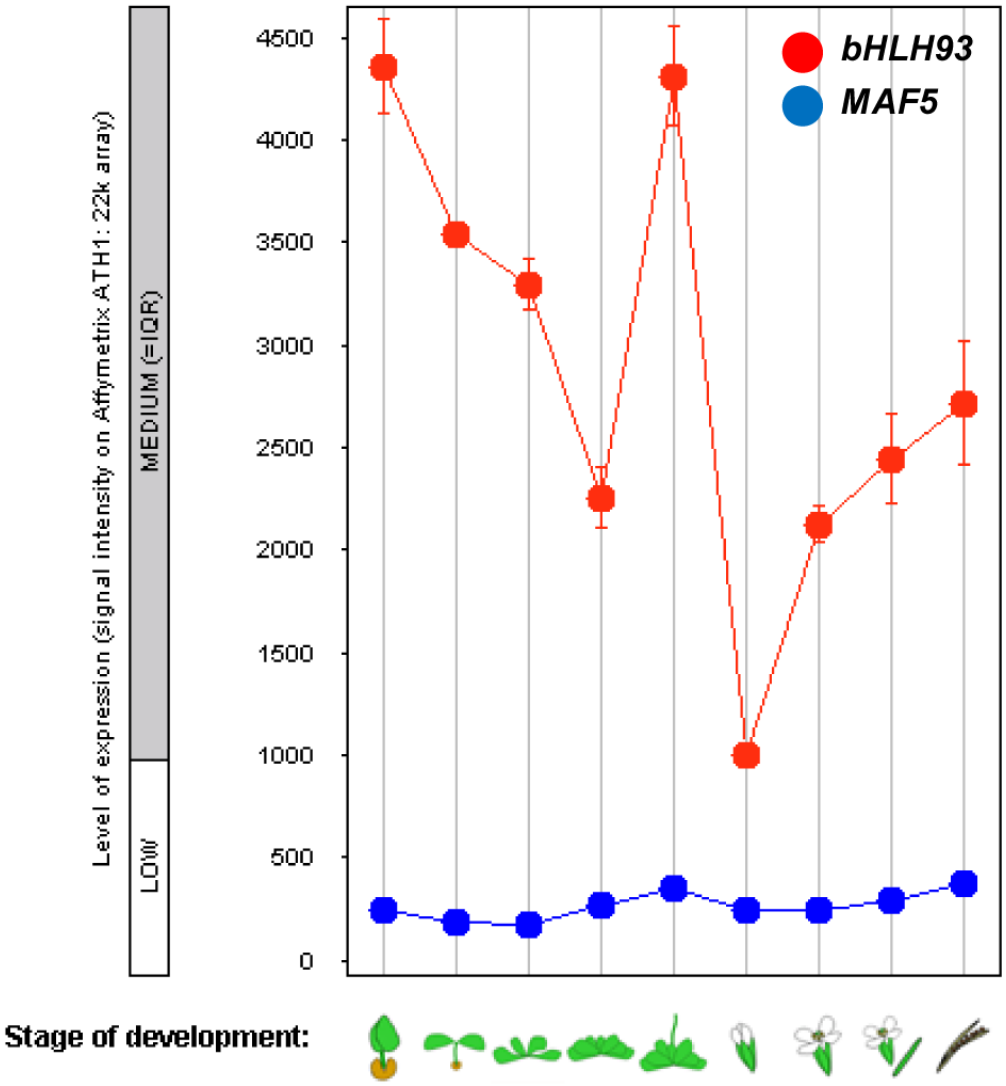

Figure 3.5: Developmental expression pattern of *bHLH93* and *MAF5*.

Digital expression patterns for *bHLH93* and *MAF5* were obtained from Genevestigator website.

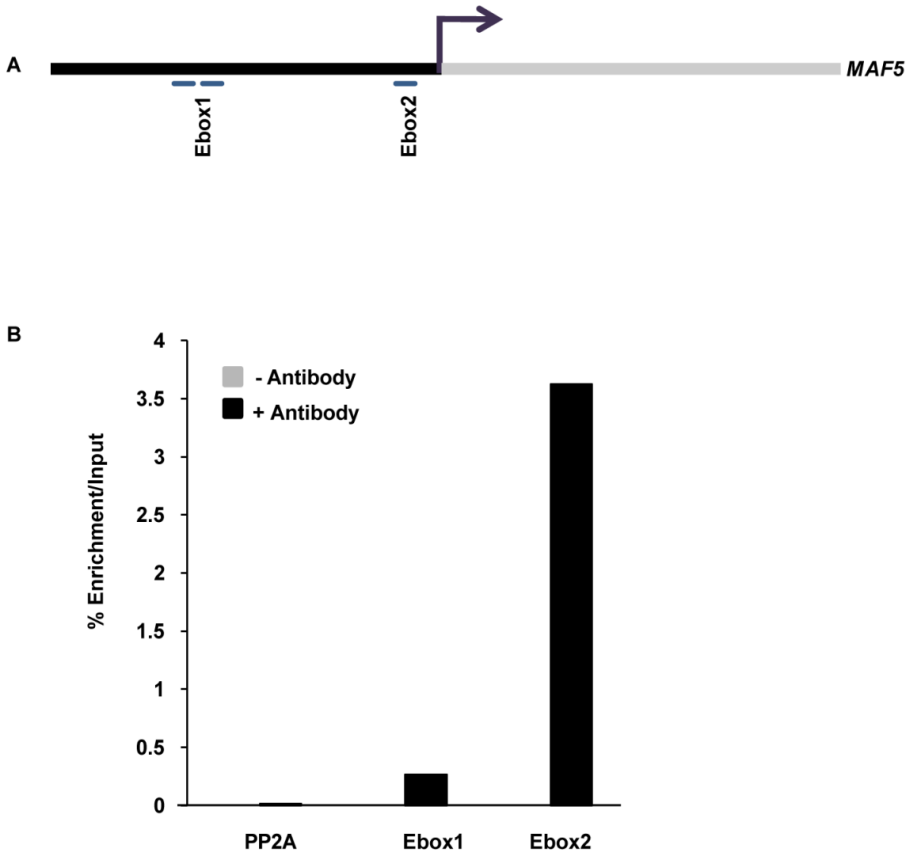

Figure 3.6: bHLH93 binds to *MAF5* promoter.

A) Diagrammatic representation of the *MAF5* gene structure showing bHLH93 binding sites. Two E-boxes are closely placed upstream and was named E-box 1. Third E-box is closer to transcription start site and was named E-box. Black arrow represents transcription start site of *MAF5* gene. 2. B) Chromatin Immunoprecipitation (ChIP) assay using *pbHLH93:bHLH93:GUS* transgenic plants. Chromatin was pulled down using anti-GUS antibody and quantitative RT-PCR was done using primers specific to E-box 1 and 2. Meristem tissue from 54 days old *pbHLH93:bHLH93:GUS* SD grown plants was harvested at the end of dark period.

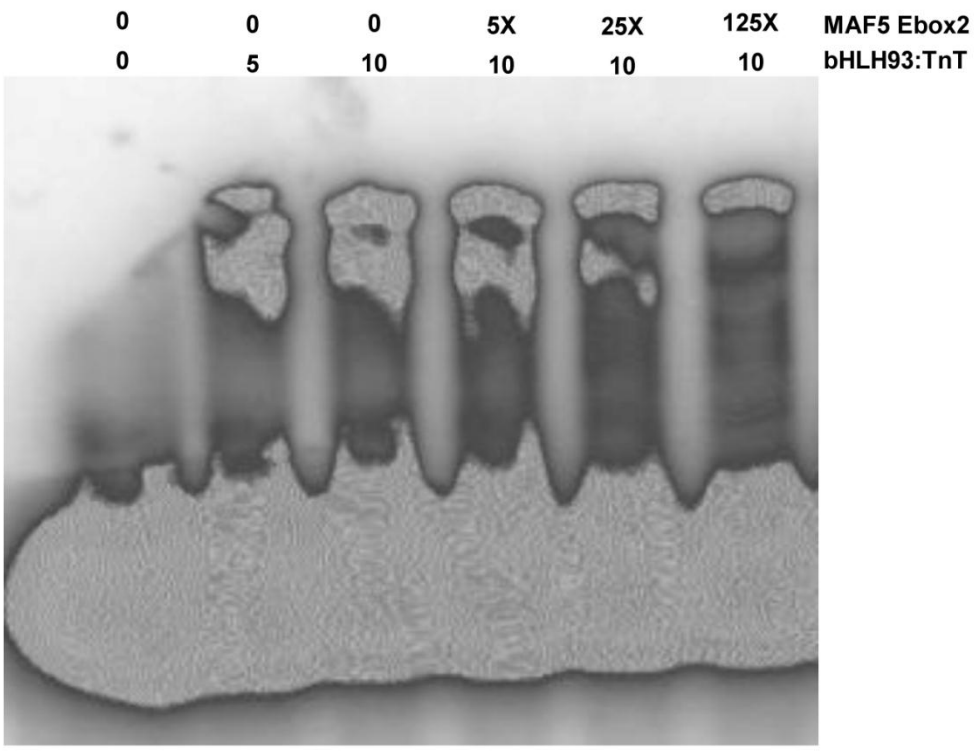

Figure 3.7: bHLH93 directly binds *MAF5* promoter.

bHLH93 protein was expressed in TnT expression system and *MAF5* promoter fragment containing E-box 2 region was used as a probe. *MAF5* wt probe was used for binding competition.

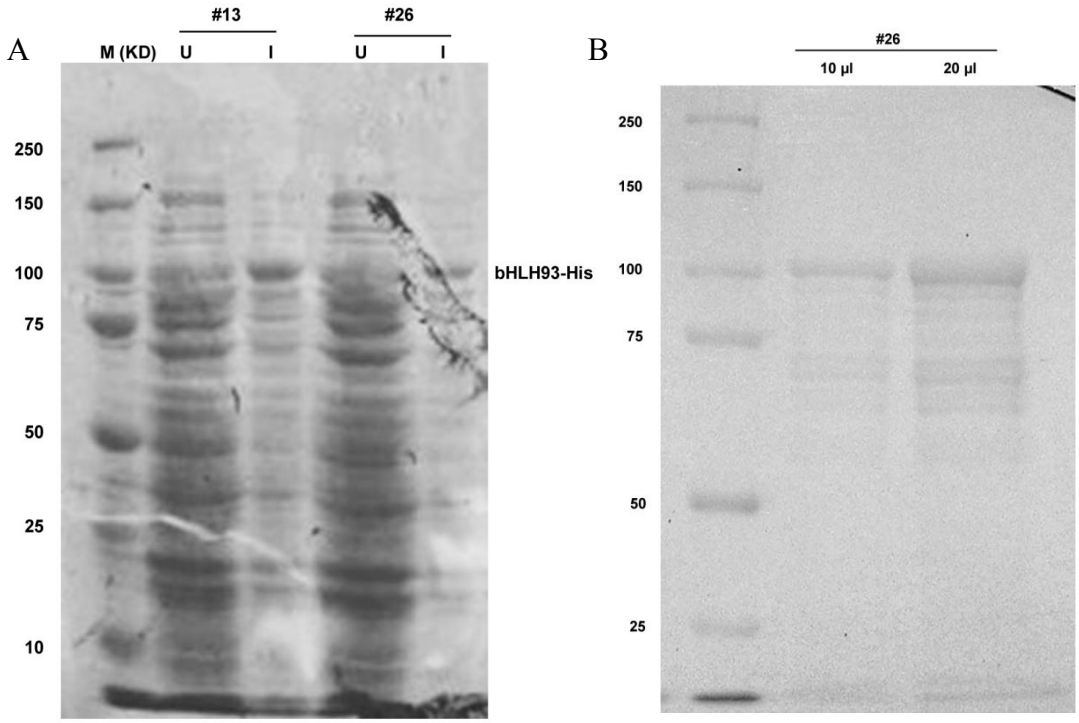

Figure 3.8: SDS-PAGE gel showing recombinant bHLH93-His protein from *E. coli*.

bHLH93 (39 KD) was cloned into pCold vector using EcoR1 restriction sites. pCold vector has 6X His tag at N-terminus. Two independent clones (#13, #26) of recombinant bHLH93-His protein (98KD) were transformed into Solubl21competent cells.A) Protein was induced using 1mM IPTG at 26^{o}C overnight. U=Un-induced total protein, I=Induced total protein, M=Protein marker. B) Purified protein from clone #26. His-bHLH93 protein is seen as a distinct band on SDS-PAGE gel stained with Coomasie Blue stain.

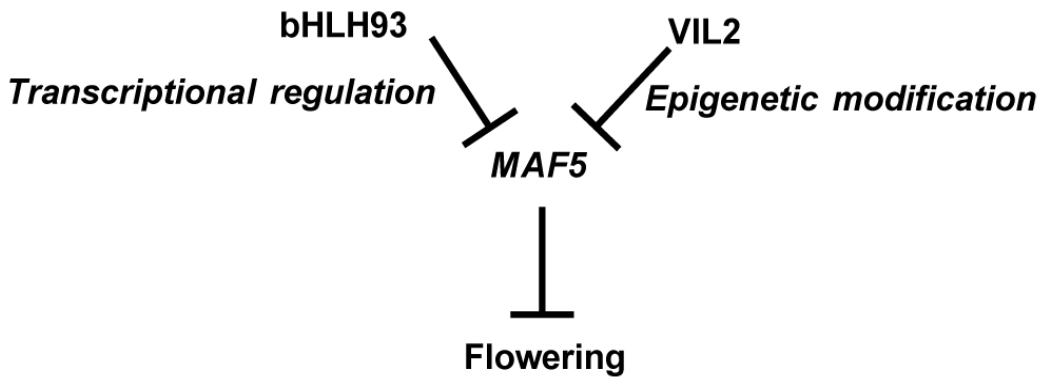

Figure 3.9: Model representing a novel mechanism of regulation of *MAF5* expression by bHLH93.

VIL2 has been shown to regulate *MAF5* by epigenetic modification. bHLH93 regulates *MAF5* expression by directly binding to the MAF5 promoter and bHLH93 act as transcriptional repressor.

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